User Manual for HoloStudio M4 2.5

with HoloMonitor M4

Phase Holographic Imaging

HoloStudio M4 2.5

Software instruction manual

©2013 Phase Holographic Imaging AB

Contact us:

Phase Holographic Imaging
Scheelevägen 22
SE-223 63 Lund
Sweden

+46-(0)46-386080

info@phiab.se

More information can be found on our webpage: www.phiab.se

Introduction to HoloMonitor™ M4

The HoloMonitor™ M4 is an incubator proof time-lapse and cell tracking instrument for adherent cells. It can capture and analyze adherent cells for number, movement, area and thickness.

The HoloMonitor™ M4 uses digital holography. This technique is based on how the cells shift the phase of light that passes through the cells. Based on how the cells affect the light, a cell image is reconstructed.

This kind of live cell imaging does not require any kind of labeling or staining.

Introduction to Holostudio 2.5

HoloStudio™ M4 is a software especially designed to capture and analyze digital holographic images with the HoloMonitor™ M4. There are two versions of the software, with or without the tracking functions.

HoloStudio™ M4 2.5 enables the user to capture and analyze images both as single captures and as time-lapse captures.

The procedure is simple: the user captures images of the sample using the HoloMonitor™ M4, and the images are analyzed in the software HoloStudio™. The imaging procedure does not affect the cells in any measurable way.

To simply count cells takes less than one minute. Confluence measurements as well as area and volume calculations are performed simultaneously. Tracking cells through a timelapse sequence is only a click away.

Table of Contents

HoloStudio M4 Tracking outline **10**
Main tabs overview **10**
Live Capture **10**
Main Viewing window **11**
Side windows **11**
Software Manual **11**

Manual PART ONE, Quickguides **12**
Time lapse capture **13**
Cell counting and confluence **14**
Volume and area analysis **15**
Cell tracking **16**

Manual PART TWO, a user guide **17**

1. Startup **17**
1.1. Startup **17**
1.2. Close down **17**

2. View live images **18**
2.1. View a live holographic image **18**
2.1.1. Focus the holographic image *18*
2.1.2. Change the holographic image display *19*
2.1.3. Move, flip or zoom the holographic image in the Main Viewing Window *20*
2.1.4. Change the holographic image coloring *20*

3. Capture images **22**
3.1. Store captured images **22**
3.2. Capture a single image **22**
3.3. Capture a time lapse sequence **22**

4. View captured images **24**
4.1. Select an image **24**
4.2. Move, flip or zoom the cell image **24**
4.3. Holographic image display **24**
4.3.1. Change the image display *24*
4.3.2. Change the image coloring *26*
4.4. Move, flip or zoom the cell image **27**
4.5. Recalculate a holographic image **27**
4.5.1. Recalculate the focus automatically *27*
4.5.2. Recalculate the focus manually *27*
4.5.3. Recalibrate the image *28*
4.5.4 Using a background hologram *28*

5. Cell identification **29**
5.1. Identify cells **29**
5.1.1. Select an image *29*
5.1.2. Automatic threshold settings *29*
5.1.3. Adjust the cell identification *30*
5.2. Make adjustments for single cells **31**
5.3. Save the cell identification settings **32**
5.4. Change the image display **33**

6. Cell Count and Analysis **34**
6.1. Measure distances directly in the currently viewed image **34**
6.2. Count cells **34**

6.3. Adjust the histogram proportions **36**
6.4. Remove data from plot **36**
6.5. Export results **36**

7.Cell Tracking **37**
7.1. Tracking cell movement **37**
7.1.1 Adding frames to the analysis *37*
7.1.2. Adding cells *37*
7.1.3. Displaying the cell tracking *38*
7.2. Adjusting the cell tracking **38**
7.2.1. Select the cell to be adjusted *38*
7.2.2. Switch the tracking from one cell to another *38*
7.2.3. Discontinue a cell tracking *39*
7.2.4. Continue a discontinued cell tracking *39*
7.2.5. Undo manual changes *39*
7.3. Export the tracking results **39**

8. Export images and movies **40**
8.1. Add and remove image frames **40**
8.2. Edit the images **41**
8.2.1. Zoom, move or flip the image *41*
8.2.2. Adjust the image display *41*
8.2.3. Holographic image coloring *42*
8.3. Create an AVI movie **43**
8.4. Export images **43**

Manual PART THREE, a list of functions **44**
HoloStudio M4 Outline **44**
The Main tabs **44**
The Main Viewing window **45**
The Side windows **45**

9. Live Capture **46**
9.1 Presets **46**
9.2. Viewer Options **46**
9.3. Coloring **48**
9.4. Software Focus **49**
9.4.1. Automatic focus *49*
9.4.2. Manual focus *50*
9.4.3. More *50*
9.4.4. Calibrate Objctive *50*
9.5. Camera Properties **50**
9.6. Calibration **51**
9.7. Capture **51**
9.7.1. Capture a single image *52*
9.7.2. Capture a timelapse *52*

10. View Images **53**
10.1. Viewer Options **53**
10.1.1. Buttons in Viewer Options *53*
10.1.2. The Measure Button *54*
10.1.3. The display options in the Viewer Options window *54*
10.2. Coloring **55**
10.3. Software Focus **56**
10.3. Software Focus **56**
10.3.1. Automatic *57*
10.3.2. Manual *57*
10.3.3. More *58*
10.4. Calibration Settings **58**
10.5. Image Presentation **58**

10.5.1. Image information *59*
10.5.2. Check and Delete *59*
10.5.3. Image display *60*

11. Identify Cells **61**
11.1. Viewer Options **61**
11.2. Advanced **61**
11.3. Adjustments **62**
11.3.1. Background Threshold *62*
11.3.2. Object Definition *64*
11.3.3. Miscellaneous *64*
11.4. Stored Settings **64**
11.5. Manual Changes **64**
11.6. Image Frame list **65**
11.6.1. Image information *65*
11.6.2. Check and Delete *66*
11.6.3. Image Display *66*
11.6.4. Save changes *66*

12. Cell Count **68**
12.1. The Cell Count Report **68**
12.1.1. Create a Cell count report *68*
12.1.2. The contents of a cell count report *68*
12.2. Growth Area and Volume text boxes **69**
12.3. Save Report **69**
12.4. The Source Frames list **69**
12.5. The Remove buttons **69**
12.6. Image Frame list **69**
12.6.1. Image information *70*
12.6.2. Check and Delete *70*
12.6.3. Image display *71*
12.7. The Add buttons **71**
12.8. The Clear All button **71**

13. Cell Tracking **72**
13.1. The Tracking tab **72**
13.1.1 Adding frames to the analysis *72*
13.2. The Select Mode side window **73**
13.2.1. Add cells *73*
13.2.2. Select cells *74*
13.3. The Change Tracking side window **74**
13.3.1. Modify Location *74*
13.3.2. Unset *74*
13.3.3. Set location *74*
13.3.4. Undo for this frame *75*
13.3.5. Undo for all frames *75*
13.3.6. Delete *75*
13.4. Track **74**
13.5. Export **74**
13.6. Identify **74**
13.7. Save **75**
13.8. Center **76**
13.9. Timeline **76**
13.10. Plot Movement Tab **76**
13.10.1. Spatial Tracking diagram *76*
13.10.1. Spatial Tracking diagram *76*
13.11. Source frames tab **78**
13.11.1. Delete frames *78*
13.12. Tracked cells tab **78**
13.12.1. Naming individual cells *78*
13.12.2. Deleting cells *78*

14. Export Images 79
14.1. Viewer Options 79
14.2. Perspective 80
14.3. Coloring 80
13.4. Main Viewing window 82
14.5. Export 82
14.5.1. Export Images 82
14.5.2. Export Movie 82
14.6. Preview 83
14.7. Remove 83
14.8. Image Presentation 83
14.8.1. Image information 84
14.8.2. Check and Delete 84
14.8.3. Image display 84
14.9. Add frames 85

15. Main top menu 86
15.1. File 88
15.1.1. Projects 86
15.1.2. Groups 86
15.1.3. Exit the program 86
15.2. View 86
15.3. Database 87
15.3.1. Database settings 87
15.3.2. Database import 87
15.3.3. Database export 88
15.4. About 88

Manual PART FOUR 89
Chapter 16. Troubleshooting 89
16.1. Focusing 89
16.1.1. The Live Capture tab is inactive 89
16.1.2. It is impossible to focus the live image 89
16.1.3. The cells are very bright and blurry showing no inner structures 89
16.1.4. The cell image is completely white 89
16.1.5. The cell image is black 89
16.1.6. The live image focus was OK, but it slowly turned bad and now it can not be set again 90
16.2. Capture 90
16.2.1. The cell image in the Main Viewing window is white 90
16.2.2. It is impossible to capture an image as the capture button is inactive 90
16.2.3. In a series of captured images not all images were good 90
16.3. View Images 90
16.3.1. No image frames are visible in the The Image Frame list 90
16.3.2. The cell image in the Main Viewing window is white 90
16.4. Cell Identification 90
16.4.1. No image frames are visible in the The Image Frame list 90
16.4.2. The cell image in the Main Viewing window is white 91
16.4.3 The automatic cell identification looks strange 91
16.4.4. Some cells are incorrectly segmented as two or more 91
16.4.5. Two cells are segmented as one 91
16.5. Cell Count 91
16.5.1. No image frames are visible in the The Image Frame list 91
16.6. Export images and movies 91
16.6.1. No image frames are visible in the The Image Frame list 91
16.6.2. The cell image in the Main Viewing window is white 91

Index 92

HoloStudio M4 2.5 ™ outline

Main tabs overview

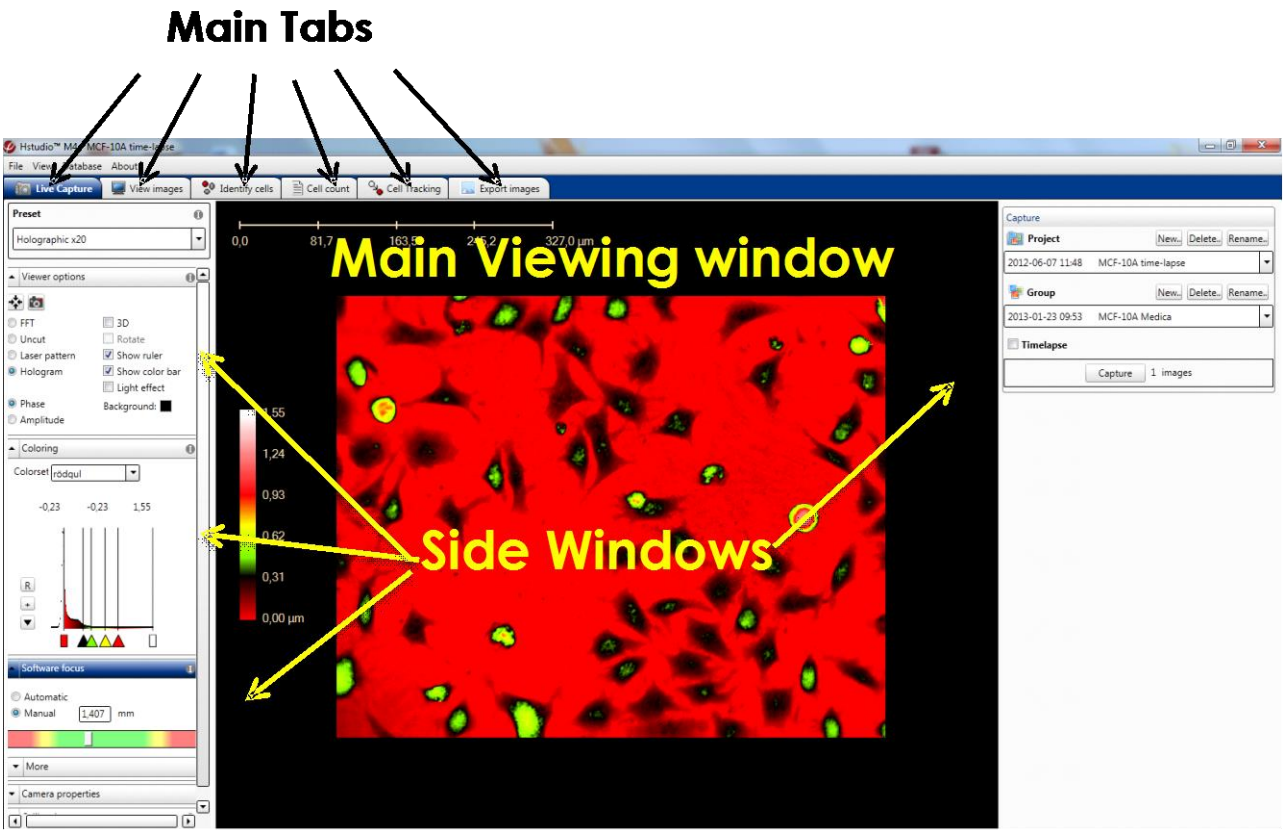

Figure 1: Overview of a main tab

HoloStudio M4 2.5 ™ is divided into six functional parts that are represented in six different tabs (Figure 1):

1. **Live Capture**, which concerns the live viewing and capturing of digital hologram and phase contrast images.
2. **View Images,** which concerns viewing captured images.
3. **Identify Cells,** which concerns the segmentation of the image resulting in the outlining and identification of the cells.
4. **Cell Count,** which concerns the analysis of the captured hologram images and display and export of the results.
5. **Cell Tracking**, which concerns the tracking of individual cells through a series of captured images.
6. **Export Images,** which concerns the visualization and export of images and movies.

The Main Viewing window

The Main Viewing window (Figure 1) shows the actual live cell image when the Live Capture tab is open and when the other tabs are open it shows the currently selected stored image.

The Side windows

Basic functions are found in side windows to the left and right of the Main Viewing window (Figure 1). If the side windows are collapsed they can be expanded by clicking the black arrow tip found in every side window header.

Additional functions or parameters are found in the More menus in some of the side windows. These functions are usually not needed for the user but rather for the service engineers.

Information concerning the different side windows can be found by clicking the Information buttons which are placed in the side headers (Figure 2).

Figure 2: Information button

The Software Manual

In the first part of the manual there are quick-guides to the most common procedures such as cell counting, timelapse captures and cell tracking.

In the second part of the manual, in chapters 1-8, there are detailed guides of how to perform different procedures using HoloStudio M4, such as how to capture an image and how to analyze the images.

In the third part of the manual, in chapters 9-15, there are detailed descriptions of all HoloStudio M4 functions in the order of appearance, from the top to the bottom and from left to right for each tab.

In the fourth part of the manual, in chapter 16, there is a trouble-shooting guide.

Manual PART ONE

Quick-guides to commonly used procedures using HoloStudio M4

Timelapse capture and export

Cell counting and confluence measurements

Volume and Area analysis

Cell tracking in a timelapse sequence (only for M4 Tracking)

Quick-guide: Time lapse capture and export using HoloStudio M4.

Startup

1. Make sure that the instrument has been placed in an incubator for at least three hours while switched on. If the instrument is to be operated at room temperature, start the HoloMonitor 30 minutes prior to use.
2. Start the computer.
3. Start HoloStudio M4.

Image capture

4. Go to the Live Capture tab.
5. Choose/create a project and a group.
6. Put the cell sample on an adapter plate of the correct type. Make sure the image is focused.
7. Calibrate the background in the calibrate menu in the left side window.
8. Activate the Timelapse function and enter the total time and the interval between the time points.
9. Press Capture.
10. Use the View Images tab to ensure that the captured images correspond to your settings. Make sure that the captured images look good.
11. When image capture is complete, proceed to the View Images tab.
12. Run through your images by clicking the Autoscroll button. If needed, recalculate the images.

Export individual images or a movie

13. Go to the Export Images tab.
14. Highlight or check the image frames you want to include in the movie or image export.
15. Click the Add buttons in the lower right corner of the program to add the data from the highlighted or checked image frames.
16. Adjust the coloring and viewing angles of the added images.
17. Preview the movie.
18. Export Images or movies.

Quick-guide: Cell counting and confluence measurements using HoloStudio M4

Startup

1. Make sure that the instrument has been placed in an incubator for at least three hours while switched on. If the instrument is to be operated at room temperature, start the HoloMonitor 30 minutes prior to use.
2. Start the computer.
3. Start HoloStudio M4.

Image capture

4. Go to the Live Capture tab.
5. Choose/create a project and a group.
6. Put the cell sample on an adapter plate of the correct type.
7. Calibrate the background in the calibrate menu in the left side window.
8. Ascertain that the live image looks well.
9. Press Capture.
10. Use the View Images tab to ensure that the captured images correspond to your settings.
11. Move the sample.
12. Press Capture. Continue until at least five images have been captured.
13. When the image capture is complete, proceed to the View Images tab.
14. Make sure that the captured images look good. Proceed to the Identify Cells tab.

Identify cells

15. Adjust the cell identification in the first image frame using the Adjustments and Manual Changes tabs.
16. Make sure all your images are checked in the Image Frame list on the right side.
17. Click the Apply checked button, which is found to the bottom right of the window, in order to apply the cell identification adjustments on all the captured images.
18. Quickly preview the rest of the images to make sure they are all well segmented. Note that for cell counting, the cell area identification does not need to be exact, but the cell markers (blue dots) need to be correctly placed. For confluence measurements the threshold setting needs to be correct.
19. Proceed to the Cell Count tab.

Acquire the cell numbers for one vessel

20. Highlight or check the image frames you want to include in the plot.
21. Click the Add buttons, which are found in the lower right corner of the window, to add the data from the highlighted or checked image frames to the cell count images list.
22. The cell count results are given as "Nbr of cells in vessel:" and the confluence results are given as % in the Cell Count Report in the main window. Type the vessel growth area in the text-box below the Cell Count Report window and choose the unit from the scroll bar to obtain a correct cell count/area, or a vessel media volume for a correct cell count/volume.
23. Export and save the Cell Count Report by clicking the Save Report button.

Quickguide: Volume and area analysis using HoloStudio M4.

Startup

1. Make sure that the instrument has been placed in an incubator for at least three hours while switched on. If the instrument is to be operated at room temperature, start the HoloMonitor 30 minutes prior to use.
2. Start the computer.
3. Start HoloStudio M4.

Image capture

4. Go to the Live Capture tab.
5. Choose/create a project and a group.
6. Put the cell sample on an adapter plate of the correct type. Make sure the image is focused.
7. Calibrate the background in the calibrate menu in the left side window.
8. Press Capture. Repeat Capture until the number of images suffices.
9. Use the View Images tab to ensure that the captured images correspond to your settings.
10. When image capture is complete, proceed to the View Images tab.
11. In the View Images tab, make sure that the images look good. Proceed to the Cell Identify Cells tab.

Cell segmentation

12. Adjust the cell identification in the first image frame using the Adjustments and Manual Changes tabs.
13. Make sure all your images are checked in the Image Frame list on the right side.
14. Click the Apply checked button to the bottom right of the program in order to apply the cell identification adjustments on all the captured images.
15. Quickly preview the rest of the images and, if needed, adjust them to make sure they are all well segmented.
16. Proceed to the Cell Count tab.

Data analysis using HoloStudio

17. Highlight or check the image frames you want to include in the analysis.
18. Click the Add buttons in the lower right corner of the program to add the data from the highlighted or checked image frames to the plots.
19. Export and save the Cell Count Report containing the area and volume by clicking the Save Report button.

Quick-guide: Cell tracking in a timelapse using HoloStudio M4

For M4 Tracking only

Startup

1. Make sure that the instrument has been placed in an incubator for at least three hours while switched on. If the instrument is to be operated at room temperature, start the HoloMonitor 30 minutes prior to use.
2. Start the computer.
3. Start HoloStudio M4.

Image capture

4. Go to the Live Capture tab.
5. Choose/create a project and a group.
6. Put the cell sample on an adapter plate of the correct type. Make sure the image is focused.
7. Calibrate the background in the calibrate menu in the left side window.
8. Activate the Timelapse function and enter the total time and the interval between the time points.
9. Press Capture.
10. Use the View Images tab to ensure that the captured images correspond to your settings.
11. When image capture is complete, proceed to the View Images tab.
12. Run through your images by clicking the Autoscroll button.

Cell segmentation

13. Adjust the cell identification in the first image frame using the Adjustments and Manual Changes tabs.
14. Make sure all your images are checked in the Image Frame list on the right side.
15. Click the Apply checked button to the bottom right of the program in order to apply the cell identification adjustments on all the captured images.
16. Quickly preview the rest of the images to make sure they are all well segmented. Adjust the segmentation if needed.
17. Proceed to the Cell Tracking tab.

Track cells through the timelapse

18. Highlight or check the image frames you want to include in the tracking.
19. Click the Add buttons in the lower right corner of the program to add the data from the highlighted or checked image frames.
20. Activate the Add cells function in the Select Mode side window. Add cells to be tracked by clicking them.
21. Follow the tracking by using the Timeline which is found below the Cell tracking window.

Spatial tracking

22. Select the Plot Movement tab, which is found behind the Tracking tab. The directions of the cell movements are given in a diagram.

Export tracking data

23. Select the Tracking tab and click Export to create an xml-file containing all the tracking data.
24. The cell tracking image containing the tracks can be saved by using the snapshot button in the Tracking tab. The spatial tracking diagrams can be exported by using the Export Plot button in the Plot Movement tab.

Manual PART TWO a user guide

For further information concerning the HoloMonitor, please use the instrument instruction manual.

Chapter 1. Startup

1.1. Startup

- Start the instrument by connecting the electrical cord. Make sure that the instrument has been placed in an incubator for at least three hours while switched on. If the instrument is to be operated at room temperature, start the HoloMonitor 30 minutes prior to use.
- Start the computer.
- Open HoloStudio.

If you want to work with HoloStudio without using the instrument:

- Start the computer.
- Open HoloStudio.

When HoloStudio is not connected to an instrument, the Live Capture tab will be inactive.

1.2. Close down by disconnecting the electrical cord and by closing the computer program.

Chapter 2. View live images

2.1. View a live holographic image

Select the Live Capture tab (Figure 3).

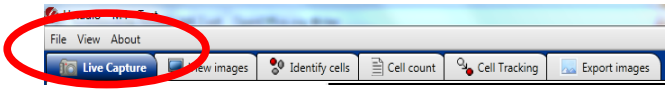

Figure 3: The Live Capture tab

Choose the correct adapter plate and put it on the stage. Then put the cell sample on the stage. An image will appear in the Main Viewing window. Calibrate the background In the calibrate menu on the left side window.

2.1.1. Focus the holographic image

Essentially, the image is focused if the correct adapter plate is used. If the image is out of focus, try a different adapter plate or a different combination of plates.

Below, three holographic phase images are shown that are in focus, out of focus and completely out of focus (Figures 4, 5 and 6).

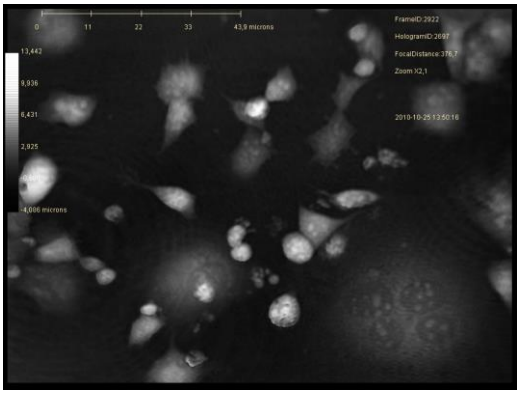

Figure 4: A digital holographic image that is in focus

Automatic focusing mostly results in well focused images. Some cell samples are more demanding and need to be focused manually.

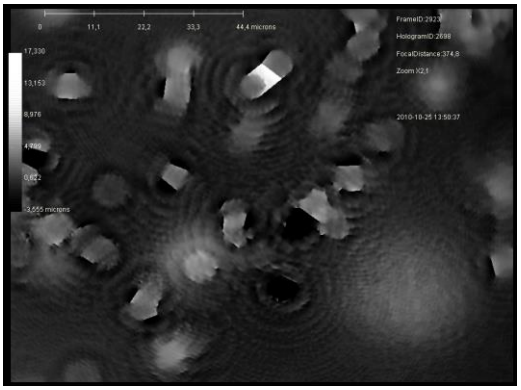

Figure 5: A holographic phase image that is out of focus

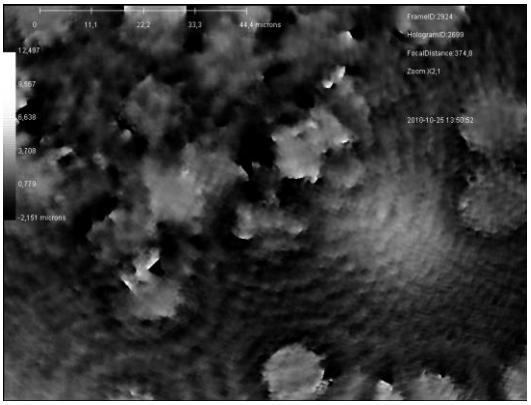

Figure 6: A holographic phase image that is totally out of focus

Check the option Manual in the Software Focus side window (Figure 7). The text box next to the Manual button shows the current software focus distance in mm. That distance can be set to a different value either by entering a value manually or by using the colored slide bar beneath the text box. Move the slide bar until the image looks good.

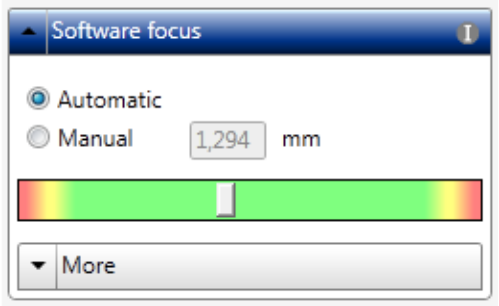

Figure 4: the Software Focus side window

As the computer updates the image with small intervals, it is recommended to await the results of one change before making further focus changes.

2.1.2. Change the holographic image display

To change how the live holographic Image is displayed, open the Viewer Options side window (Figure 8) on the left hand side of the Main Viewing window (Figure 1).

By clicking the Center button (Figure 9) the image will be centered in the Main Viewing window.

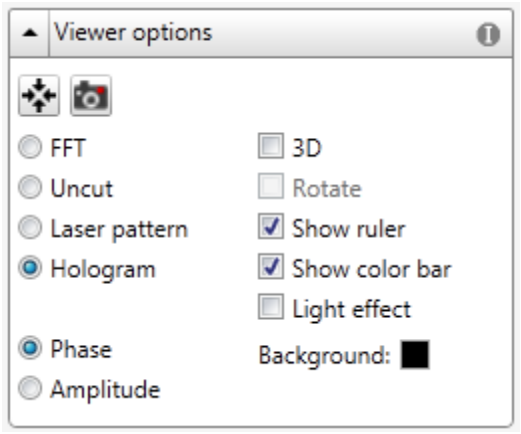

Figure 8: The Viewer Options window

Figure 9: The Center button

By pressing the Snapshot button (figure 10) an image of the current view will be saved.

Figure 10: The Snapshot button

Different display options are shown (Figure 8). They can be activated by checking the boxes. Some boxes can be checked in parallel, thus making it possible to combine functions.

Checking FFT (Figure 8) displays the Fast Fourier Transform which represents the frequency domains.

Checking Uncut displays the image as it is first reconstructed.

Checking Laser Pattern displays the original interference pattern resulting from the merging of the object and reference laser beams.

Checking Hologram displays the reconstructed image which is based on the laser pattern. The hologram can be displayed showing either the phase or amplitude information of the light wave.

Checking Phase displays the light wave phase information in the hologram.

Checking Amplitude displays the light wave amplitude information in the hologram.

Checking 3-D displays the holographic data as a 3-dimensional representation.

Checking Rotate auto-rotates the image.

Checking Show Ruler displays a horizontal scale bar representative of the distance in X and Y.

Checking Show Color Bar (Figure 8) displays a vertical scale bar representative of the height in Z.

Checking Light Effect applies an artificial light source to the image which may sometimes render an improved image.

Checking Shiny Surface applies a change in the surface image display that sometimes renders a better image. Shiny Surface is only visible when Light Effect is checked.

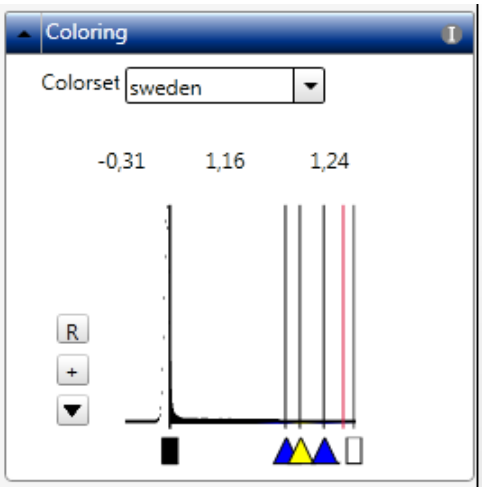

Figure 11: The Coloring side window

By left clicking the R-button (Figure 12) the coloring in the image is rescaled to better utilize the optimal dynamic range of the image. This button needs to be operated every time the image coloring is off.

2.1.3. Move, flip or zoom the holographic image in the Main Viewing Window

To zoom the live image, left click the image in the Main Viewing window (Figure 1) and then use the mouse scroll button.

To move the live image to a desired location in the Main Viewing window, click, hold and drag the image using the left mouse button.

To flip and move the live 3-D image, click, hold and drag the image using the right mouse button.

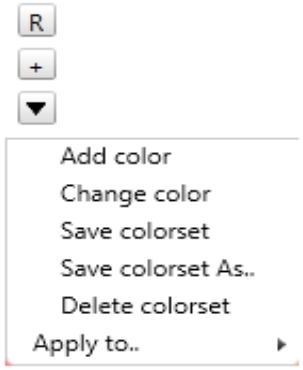

Figure 5. Coloring side window functions

2.1.4. Change the holographic image coloring

All images will appear in gray scale. If colors are needed or wanted, use the Coloring side window (Figure 11) to the left of the Main Viewing window (Figure 1).

A set of colors that are saved together is called a colorset. A previously saved colorset can be used with the current image by making a selection in the Colorset list which is found at the top of the Coloring side window (Figure 11).

A new color can be added to the colorset by clicking the plus button (Figure 12) and select a new color or by right clicking with the mouse pointer at the desired location on the axis. A colored triangle representing the new color will appear beneath the histogram (Figure 11).

Change the color by using the right mouse button to click on a colored triangle beneath the histogram and select a new color, alternatively left click the arrow button and select Change Color (Figures 11 and 12). To change the color span, left click and move the desired colored triangle beneath the histogram using the cursor (Figure 11).

To save a new colorset with the current color settings, left click the arrow button and choose Save As (Figure 12).

To save the current color settings to a previously saved colorset, left click the arrow button and choose Save (Figure 12). Note that this will overwrite the settings previously saved to this colorset.

To delete a colorset, select it in the Colorset list by left clicking it. Then left click the arrow button and select Delete Colorset (Figure 12).

Chapter 3. Capture images

Select the Live Capture tab (Figure 13).

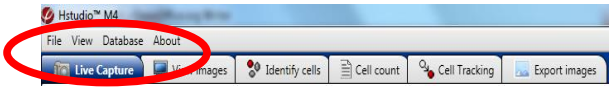

Figure 13: The Live Capture tab

Put the cell sample on the stage. A live image will appear in the Main Viewing window. Calibrate the background (left side window). Ascertain that you are satisfied with the quality of the live image (See Chapter 2).

3.1. Store captured images

Before images can be captured, a place of storage must be prepared. The images must be stored in a Group within a Project. Either open an existing project or create a new project in the Capture window (Figure 14). Then open an existing group or create a new group where the images will be saved. When a new project or group is created, the date and time are automatically included in the name.

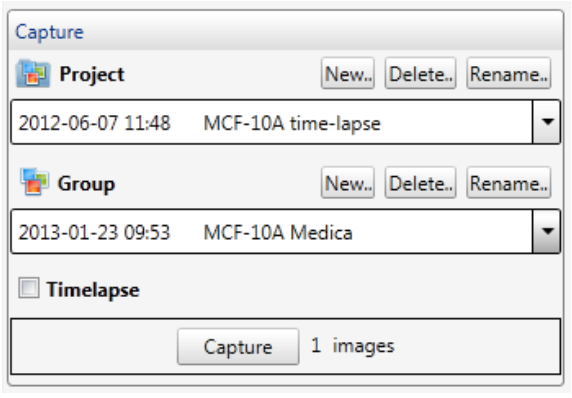

Figure 14: The Capture window

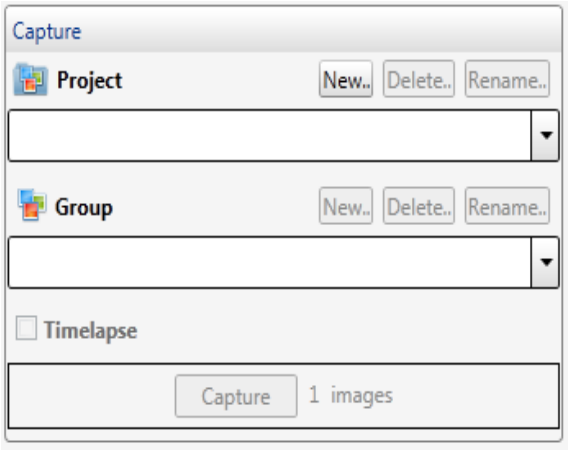

Figure 15: The Capture window with an inactive Capture button

3.2. Capture a single image

Click the Capture button.

The Capture button is inactive unless a project and a group have been selected (Figure 15).

3.3. Capture a time lapse sequence

To enable slow events to be recorded and studied, a movie can be created from images captured at intervals, *i.e.* a timelapse movie.

In order to capture images for a timelapse study, check the Timelapse box (Figure 16) in the Capture window. This box is inactive unless a project and a group have been selected.

Enter the total time for the timelapse and select the desired time unit (seconds, minutes or hours). Enter the interval between the capture time points and select the desired time unit (seconds, minutes or hours). The minimum interval is given to the right of the interval time unit box.

The number of time points will be given when total time and interval are entered.

Click the Capture button.

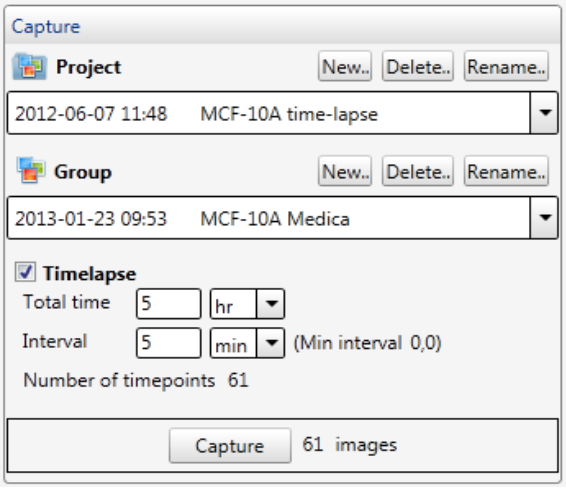

Figure 16: Activated Timelapse function

Chapter 4. View captured images

In order to view and adjust captured images, select the View Images tab (Figure 17).

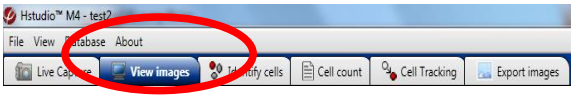

Figure 17: The View Images tab

4.1. Select an image

Start by selecting a Project and a Group (Figure 18). They are found to the right of the Main Viewing window.

An image frame list for the selected group will appear. Both holographic images and phase contrast images are presented in the list as well as information pertaining to the images. Highlighting an image will make it appear in the Main Viewing window.

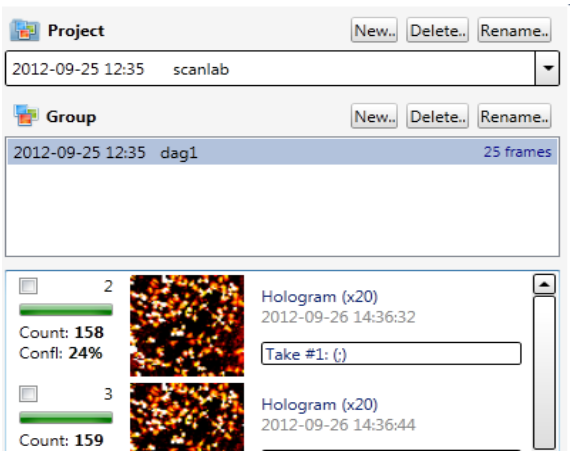

Figure 18: The Image Frame list

4.2. Move, flip or zoom the cell image

To zoom the cell image, click the image in the Main Viewing window and then use the scroll button on the mouse.

To move the cell image to a desired location in the Main Viewing window, click, hold and drag the image using the left mouse button.

To flip and move a holographic 3-D image, click, hold and drag the image using the right mouse button on the image.

4.3. Holographic image display

All holographic images will basically appear in gray scale and in 2-D unless artificial coloring has been chosen.

4.3.1. Change the image display

The holographic image display can be modulated using the Viewer Options window (Figure 19) which is found to the left of the Main Viewing window.

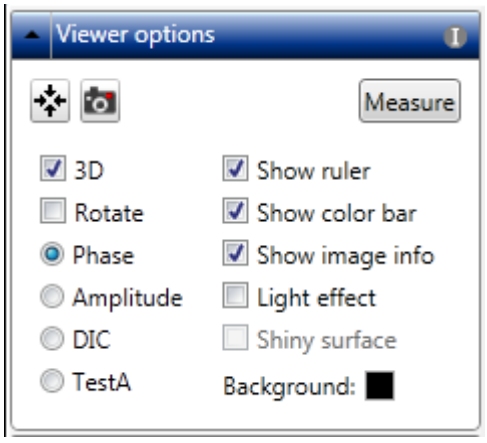

Figure 6: The Viewer Options side window

By pressing the Center button (Figure 20) the image will be centered in the Main Viewing window.

Figure 20: The Center button

By pressing the Snapshot button (Figure 21) an image of the current view will be saved.

Figure 21: The Snapshot button

By checking the boxes, different options can be activated. Most boxes can be checked in parallel to combine options.

Checking 3-D displays the holographic data as a 3-dimensional representation.

Checking Rotate auto-rotates the image.

Checking Hologram displays the reconstructed image which is based on the laser pattern. The hologram can be displayed showing either the phase or amplitude information of the light wave.

Checking Phase displays the light wave phase information in the hologram.

Checking Amplitude displays the light wave amplitude information in the hologram.

Checking Show Ruler displays a horizontal scale bar representative of the distance in X and Y.

Checking Show Color bar displays a vertical scale bar representative of the height in Z.

Checking Show Image Info displays additional information associated with the image such as (Figure 22):

The top line specifies in which project the image is located

The second line specifies in which group the image is located

Nbr: Specifies which number the image has in the group.

Type: Specifies if the image is holographic or phase

contrast.Date: specifies the capture date and time of the image

Width: specifies the image width in µm and pixels.

Height: specifies the image height in µm and pixels

Checking Light Effect applies an artificial light source to the image which may sometimes render an improved image.

Checking Shiny surface applies a change in the surface image display that sometimes renders a better image. Shiny surface is only active when Light Effect is checked.

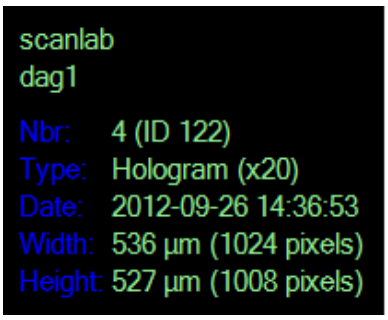

Figure 22: Hologram Image Information

4.3.2. Change the image coloring

All holographic images will appear in gray scale. Use the Coloring side window (Figure 23), which is found to the left of the Main Viewing window, to add artificial coloring to the images. The colors that are applied will be distributed in the image relatively to the thickness of the objects.

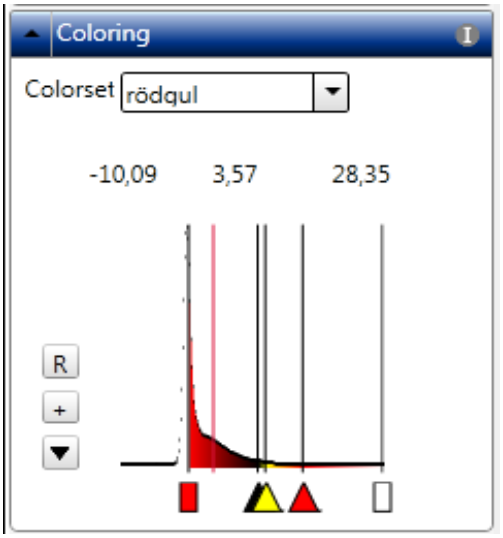

Figure 23: The Coloring side window

A set of colors that are saved together is called a colorset. A previously saved colorset can be used with the current image by making a selection in the Colorset list which is found at the top of the Coloring side window (Figure 23).

By left clicking the R-button (Figure 24) the coloring in the image is rescaled to better utilize the optimal dynamic range of the image. This button needs to be operated every time the image coloring is off.

To add a new color, left click the plus-button (Figure 24) or right click with the mouse pointer at the desired spot on the scale and click Add Color. Select a color. A colored triangle representing the new color will appear beneath the histogram (Figure 23).

Change the color by using the right mouse button to click on a colored triangle beneath the histogram (Figure 23) and select a new color. Alternatively left click the arrow button (Figure 24) and select Change Color.

To change the color span, left click and move the desired colored triangle beneath the histogram (Figure 23) using the cursor.

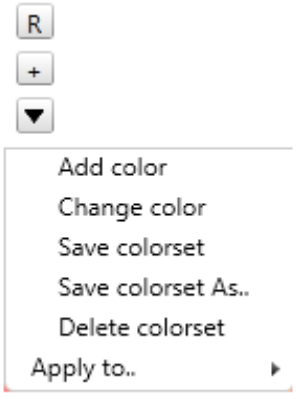

Figure 24: Additional functions found in the Coloring side window

To save the current color settings to a new colorset, left click the arrow button and choose Save As (Figure 24).

To save the current color settings to an already existing colorset, left click the arrow button and click save (Figure 24). Note that this will overwrite the settings previously saved to this colorset.

To delete a colorset, select it in the Colorset list. Then left click the arrow button (Figure 24) and click Delete Colorset.

To save a colorset to an image, left click the arrow button (Figure 24) and choose Apply To, followed by Frame. The current colorset will be applied to the currently viewed frame and saved.

To save several images with the current colorset, check the box of the desired images in the Image Presentation side window (Figure 18), left click on the arrow button in the Histogram side window (Figure 24). Choose Apply to followed by left clicking Checked Frames. The current colorset will be applied to the checked image frames and saved.

Note that changes regarding color will not in any way affect the raw data, and that by choosing default, the original grayscale image can always be retrieved.

4.4. Move, flip or zoom the cell image

To zoom the cell image, click the image in the Main Viewing window and then use the scroll button on the mouse.

To move the cell image to a desired location in the Main Viewing window, click, hold and drag the image using the left mouse button.

To flip and move a holographic 3-D image, click, hold and drag the image using the right mouse button on the image.

4.5. Recalculate a holographic image

If a holographic image is not correctly focused, as in this example (Figure 25), the software focus can be recalculated after capture in the View Images tab by changing the software calculation settings.

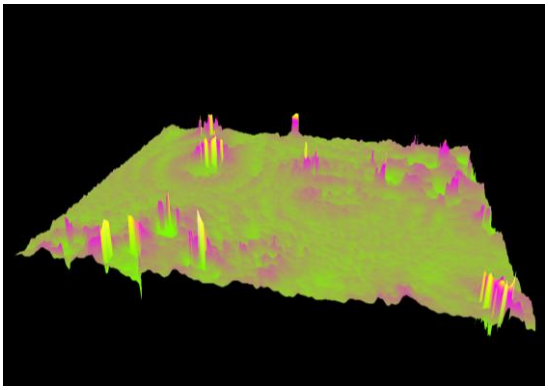

Figure 25: An unfocused holographic image

4.5.1. Recalculate the focus automatically

Select Automatic in the Software Focus side window (Figure 26) and then click the Update button. If the result is a well-focused image, use the arrow button to apply the update to either the current frame or to all checked frames.

4.5.2. Recalculate the focus manually

Automatic focusing mostly results in well focused images. Some cell samples are more demanding and need to be focused manually. Check Manual in the Software Focus side window (Figure 27).

The text box next to the Manual button shows the software focus distance in mm.

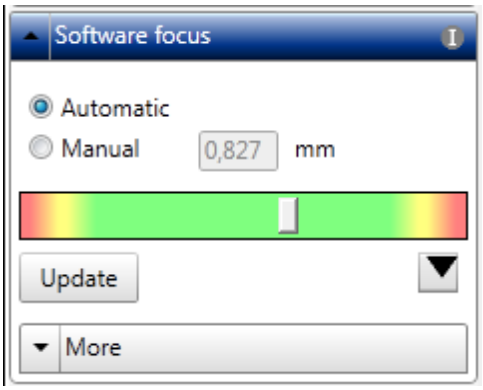

Figure 26. The Calculation Settings side window set to automatic

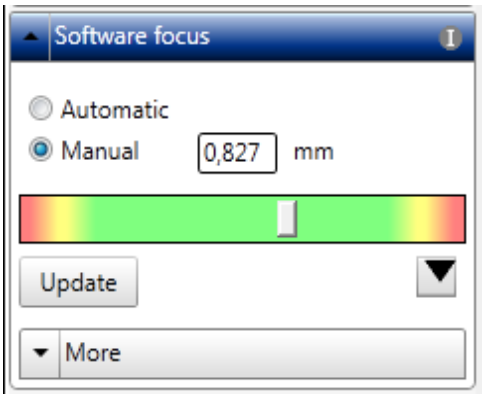

Figure 27: The Calculation Settings side window set to manual

The distance can be changed either by entering a value manually or by using the slide bar beneath the text box.

To determine which focal distance that will result in a well-focused image is often a matter of trial and error. To find a starting value, choose an image that was captured at the same time and that is well focused and note the focal distance of that image.

Highlight the image that needs to be recalculated. Select Manual in the Software Focus side window and enter the focal distance of the well-focused image in the text box.

Click Update.

If the image focus needs to be improved, enter a new focal distance and click Update. Continue until the image is well focused.

Use the arrow button to apply the update to either the current frame or to all checked frames.

4.5.3. Recalibrate the image

If a captured or imported image is not correctly centered due to aberrant calibration of the instrument, the image can be recalibrated. Clicking the Recalibrate button (Figure 28) will result in a re-centered image with an adjusted focal distance.

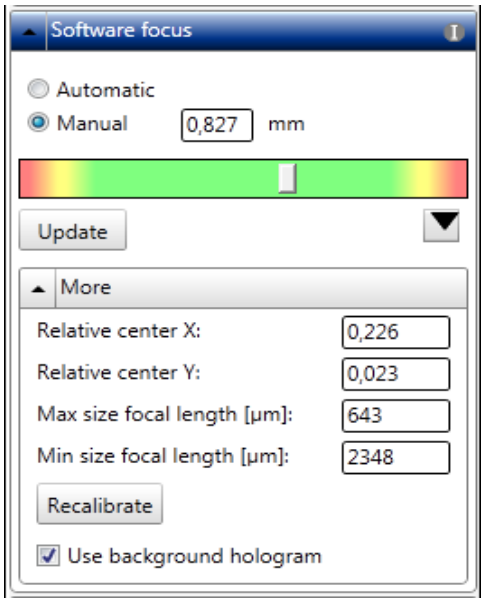

Figure 28: The More list with the Recalibration button in the Calculation settings side window

4.5.4 Using a background hologram

The images are noise-reduced by using a background hologram to subtract noise from the image. If the background hologram is not correctly set, it might instead disturb the image calculations. There is an option not to use the background hologram (Figure 28).

Chapter 5. Cell identification

The base for all image analysis is the cell identification step. In this step the image background and the cell outlines are set. Only thereafter the program can determine cell properties using the images. When the cell identification has been performed, the cell number and confluence of each image is immediately given beside the image in the Image Frame list (Figure 30).

Note that the software will suggest a default cell identification at the time of capture. This automatic identification needs to be evaluated for each sample.

Choose the Identify Cells tab (Figure 29).

Figure 29: The Identify Cells tab

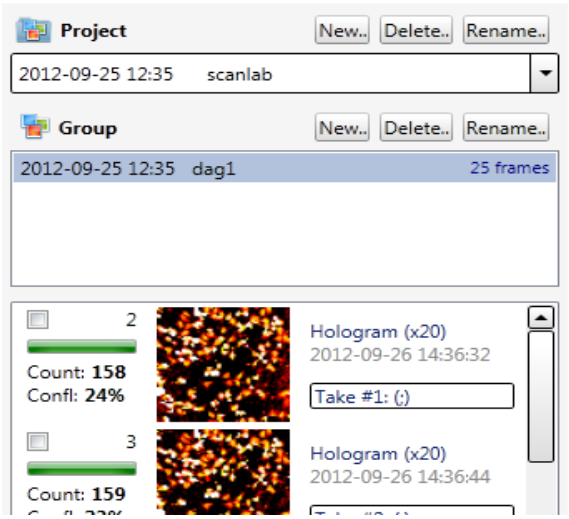

Figure 30: The Image Frame list

5.1. Identify cells

5.1.1. Select an image

The Image Frame list (Figure 30) is found to the right of the Main Viewing window (Figure 1).

Select the project and group where the images are saved (Figure 30). Highlight the image of interest to make it appear in the Main Viewing Window.

5.1.2. Automatic threshold settings

The software will automatically make threshold settings according to the default segmentation method (Figure 31). The resulting cell identification may be very good or may need adjustments (Figure 32).

There are several other methods to calculate the threshold settings in the Methods list in the Adjustments window (Figure 31) which is found below the Main Viewing window.

The different threshold settings calculation methods (Figure 31) will result in slightly different cell identifications. It is advisable to try out which method that works best for each type of cell sample.

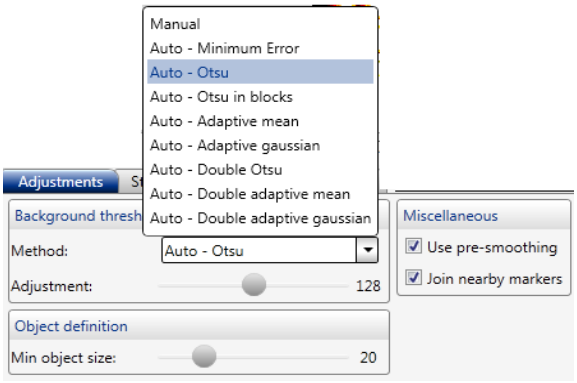

Figure 31: The Adjustments tab showing the different methods to calculate threshold settings

Manual allows the user to set the global threshold level using the slider.

Minimum error sets the global threshold level using the minimum error histogram-based threshold method.

Otsu sets the global threshold level using the Otsu method.

Otsu in blocks: the image is split into blocks which are thresholded separately using Otsu method. This is a form of adaptive threshold.

Adaptive mean sets an adaptive thresholding using a mean filter

Adaptive gaussian sets an adaptive thresholding using a gaussian filter.

Double otsu: double thresholding is a method where both a wide and a narrow threshold mask is used. The narrow image is morphologically reconstructed under the wide image. The final image is used as threshold mask. The result is a cleaner threshold mask. The Double Otsu uses double thresholding with Otsu global threshold as mid-level threshold.

Double adaptive mean: same as Double Otsu but with two adaptive mean threshold masks.

Double adaptive gaussian: same as Double Otsu but with two adaptive gaussian threshold masks.

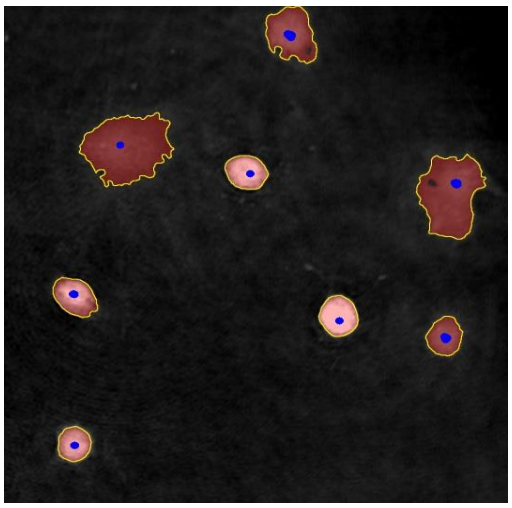

Figure 32: Identified cells

5.1.3. Adjust the cell identification

In the Adjustments tab, which is found below the Main Viewing window (Figure 33), the cell identification settings can be adjusted in several ways. In addition to selecting the method to calculate the threshold, as described above, adjustments can be made for each method.

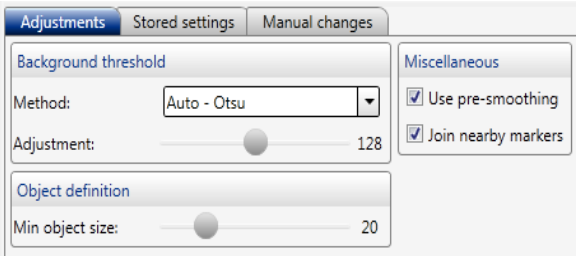

Figure 33: The Adjustments tab

The slide bar labeled Adjustment (Figure 33) is used to manually adjust the threshold that is set between cells and background, thus adjusting the area of the segmented cells.

The slide bar labeled Minimum Object Size (Figure 33) is used to manually adjust the size of the cell core, thus adjusting which identified areas that are cells.

Checking Presmoothing (Figure 33) activates a noise reduction function that will smooth the edges of the cells.

Checking Join Nearby Markers (Figure 33), result in two distinct cell markers being counted as one when they are very close to each other.

5.2. Make adjustments for single cells

It is possible to make manual changes to the cell identification for individual cells. It is possible to add, remove and delete as well as enlarge or shrink identified cells. These functions are found in the Manual Changes tab (Figure 33) below the Main Viewing window.

Several of these functions are found as a menu when clicking the right mouse button in the image (Figures 35 and 36).

To the right in this window there are buttons that enable the user to undo the last adjustment step, to redo the undone step and to clear all adjustments.

Figure 35: The adjustments menu when clicking outside identified cell areas

Add or Remove (Figure 34) allows the user to add or remove the blue cell markers that identify the cell core. This results in mergers or splits of identified cell areas.

Grow or Shrink allows the user to enlarge or shrink the identified cell regions.

Delete Area allows the user to remove identified cell areas.

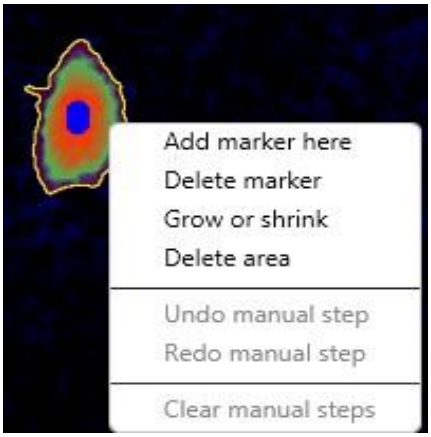

Figure 36: The adjustments menu when clicking on a cell area

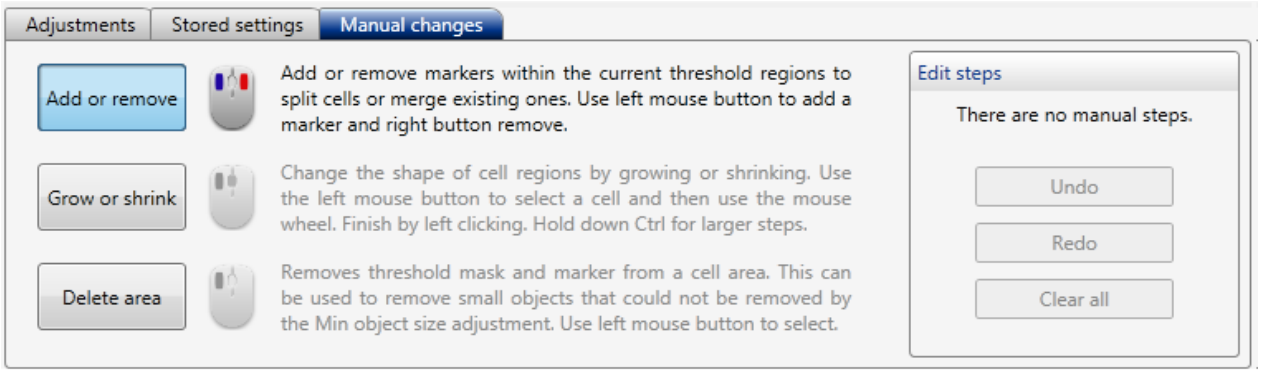
Adjustments
Stored settings
Manual changes
Add or remove
Add or remove markers within the current threshold regions to split cells or merge existing ones. Use left mouse button to add a marker and right button remove.
Grow or shrink
Change the shape of cell regions by growing or shrinking. Use the left mouse button to select a cell and then use the mouse wheel. Finish by left clicking. Hold down Ctrl for larger steps.
Delete area
Removes threshold mask and marker from a cell area. This can be used to remove small objects that could not be removed by the Min object size adjustment. Use left mouse button to select.
Edit steps
There are no manual steps.
Undo
Redo
Clear all

Figure 34 The Manual Changes tab

5.3. Save the cell identification settings

All changes are saved automatically if the box for Auto-apply Changes (Figure 37) is checked. This function is found to the left of the Main Viewing Window.

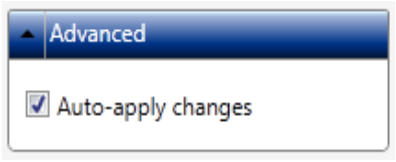

Figure 37: The Auto-apply Changes side window

If the box is not checked, a warning will appear that the changes are not saved when the user switches from the Identify Cells tab to another main tab (Figure 38).

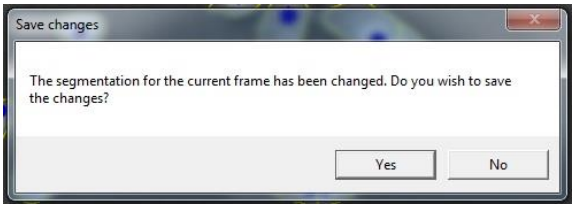

Figure 38: Warning message for changes that have not been saved

The segmentation settings can be applied to a certain frame or all checked frames or to all frames by using the Apply Current, the Apply Checked or the Apply All buttons that are found below the Image Frame list (Figure 39).

Note that changes performed using the Manual Changes window (Figure 37) are applied only to individual cells. These changes cannot be applied to other image frames.

The segmentation settings can be stored for later use in the Stored Settings tab (Figure 40), which is found below the Main Viewing window. The settings will automatically be dated and named New Presets. The name can then be changed by highlighting the preset and using the Rename button. The stored settings can be applied to an image with the Load button.

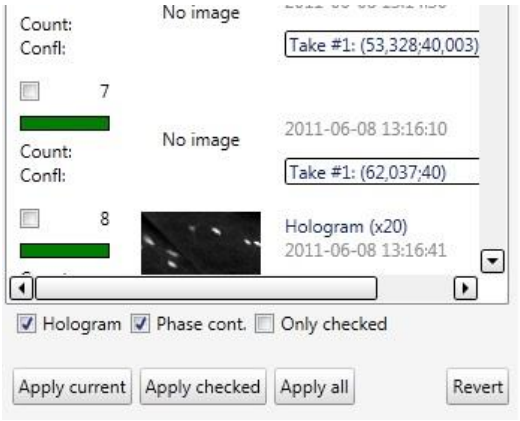

Figure 39: The Apply buttons

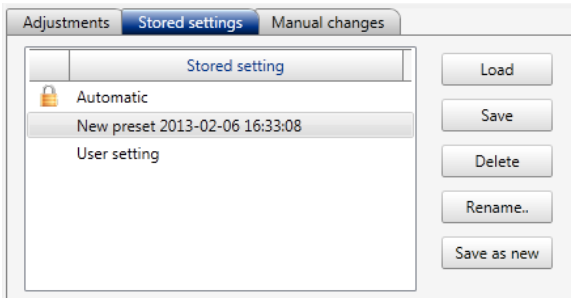

Figure 40: The Stored Settings tab

5.4. Change the image display

In the Viewer Options side window (Figure 41), which is found to the left of the Main Viewing window, it is possible to change how the image is displayed. The different display functions can be activated by checking the boxes. The boxes can be checked in parallel, enabling the user to combine functions.

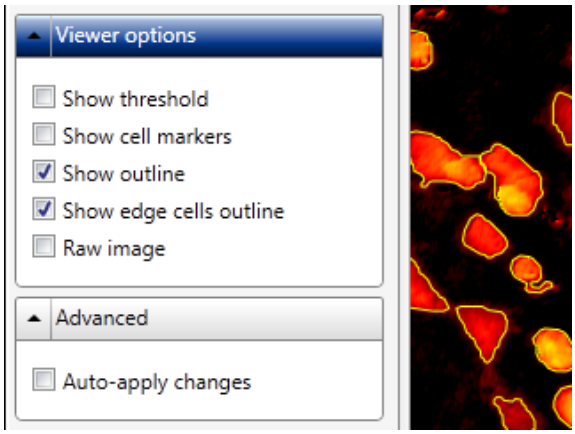

Figure 41 The Viewer Options side window

Checking Show Threshold displays a red coloring that distinguishes cells from the background.

Checking Show Cell Markers displays a blue coloring that indicates the cell core.

Checking Show Outline displays yellow lines that indicate the border of the cells.

Checking Show Edge Cells Outline displays yellow lines that indicates the border of the cells touching the image edges.

Checking Raw Image causes the image to be displayed as the unadulterated gray scale image

Using Auto-apply Changes allows the user to implement all changes immediately.

Chapter 6. Cell Count and Analysis

A tool for measuring distances or objects in a currently viewed image is found in the View Images tab.

The cells are counted and analyzed for confluence, cell area and volume in the Cell Count tab.

6.1. Measure distances directly in the currently viewed image

Choose the View Images tab (Figure 42).

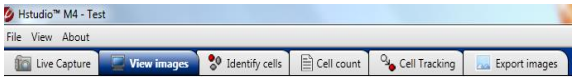

Figure 42: The View Images tab

Click the Measure button in the Viewer Options side window (Figure 43) to make manual measures of objects in the current image.

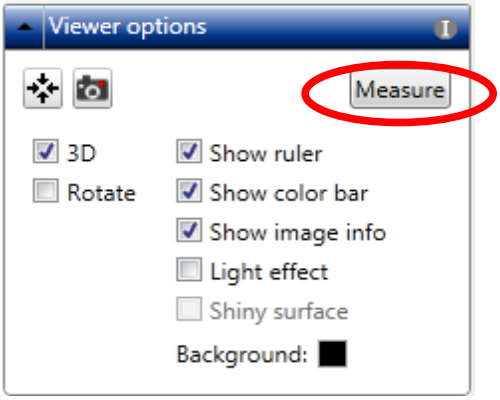

Figure 43: The Viewer Options side window for holographic images

When the measuring function is activated, the measurement is started by left clicking anywhere in the image. The measurement is finished by left clicking at a new point in the image.

The measured distance is shown with a blue bar (Figure 44). A window displaying the results appear to the bottom right of the main viewing window. The results include a profile of the measured object. The maximum thickness of the object is shown as red text by the dashed line.

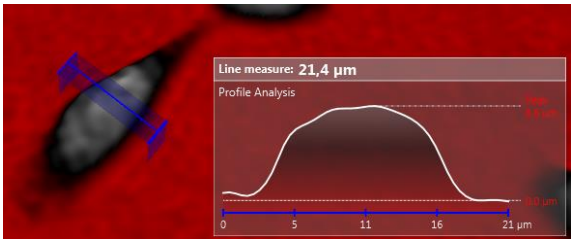

Figure 44: The blue measuring bar is seen on top of the left cell, and the results are shown in a results window

The data are saved as an image when the Snapshot button (Figure 45) in the Viewer Options window is clicked.

Figure 45: The Snapshot button

It is possible to move the image in the Main Viewing window while the measuring function is activated. The image is moved by clicking and dragging.

When the Measure button is clicked again, the measuring function is deactivated.

6.2. Count cells

Choose the Cell Count tab (Figure 46).
A text will appear which says that five image

Figure 46 The Cell Count tab

frames or more must be added in order to

analyze images for cell count, confluence, cell area and volume (Figure 47).

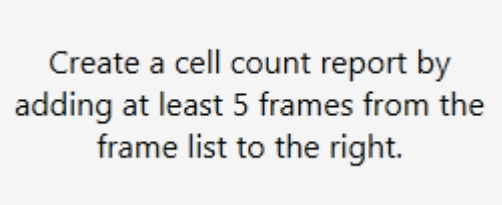

Figure 47: The Cell Count instruction text

Highlight or check the images to be analyzed, in the Image Frame List to the right. Click the appropriate Add button (Figure 48). The Add buttons are found below the Image Frame List. The added image frames will be shown in the Source Frames window below the Cell Count Report (Figure 49).

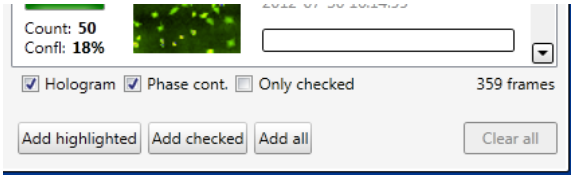

Figure 48: The Add buttons

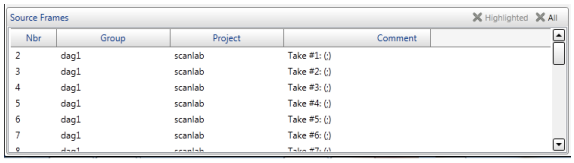

Figure 49: The Source Frames window

The images are then analyzed, and the results are presented in a Cell Count report (Figure 50).

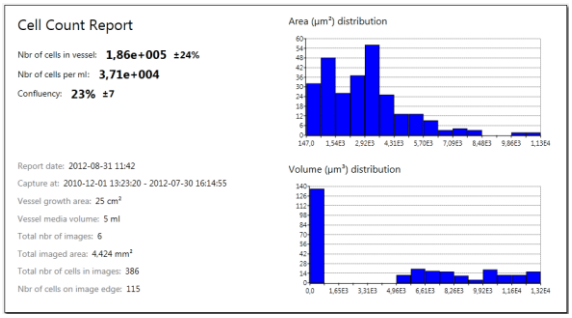
Cell Count Report
Nbr of cells in vessel: 1,86e+005 ±24%
Nbr of cells per ml: 3,71e+004
Confluency: 23% ±7
Area (µm²) distribution
Report date: 2012-08-31 11:42
Capture at: 2010-12-01 13:23:20 - 2012-07-30 16:14:55
Vessel growth area: 25 cm²
Vessel media volume: 5 ml
Total nbr of images: 6
Total imaged area: 4,424 mm²
Total nbr of cells in images: 386
Nbr of cells on image edge: 115
Volume (µm³) distribution

Figure 50: The Cell Count report

Fill in the correct cell culture vessel growth area and the volume of the cell culture vessel medium content in the text boxes below the Cell Count Report (Figure 51).

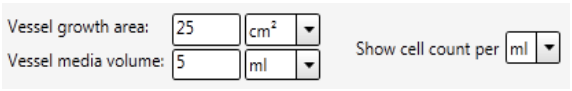

Figure 51: Text boxes for cell culture vessel area and volume

The report contains data on:

- Number of cells in the vessel
- Number of cells per ml
- Confluence
- Report date
- Capture time points
- Vessel growth area
- Vessel media volume
- Total number of image frames used for the analysis
- The total area of the images
- The total number of cells in the images
- The number of cells that are placed on the image edge, and which are therefore not included in the morphological analysis, although they are included in the cell count.

6.3. Adjust the histogram proportions

The X-axis of the Area and Volume histograms can be set either automatically, or manually. For each axis the lowest and the highest value can be set as well as the number of bins that present the data.
The adjustment text boxes are found below the cell count report window (Fig. 52).

Figure 52: The histogram adjustment functions

6.4. Remove data from plot

To clear the plot from all data from all image frames, use the Remove All command, which is found with an X to the right in the Source Frames window (Figure 53).

To remove data belonging to a single frame from a plot, highlight that frame in the Source Frames window and then use the Remove Highlighted command, which is found with an X to the right in the Source Frames window (Figure 53).

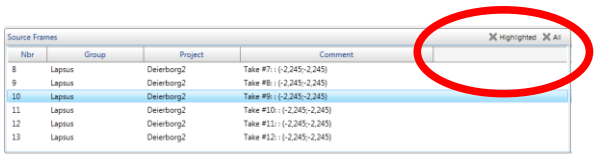

Figure 53: The Remove functions

6.5. Export results

Below the Cell Count Report there is a Save report button (Figure 54). Clicking the button generates a pdf file containing all relevant data, including the area and volume as histograms and a list of the included image frames. When the boxes are un-checked, histograms and frame list can be excluded from the report.

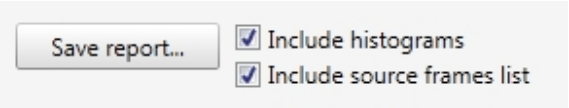

Figure 54: The Save Report button

Chapter 7.Cell Tracking

For M4 Tracking only

Cells can be tracked through a timelapse sequence and analyzed both for movement and for morphology changes over time.

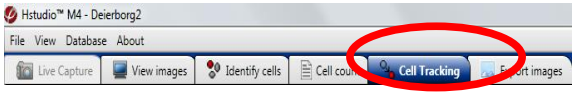

Figure 7: The Cell Tracking tab

7.1. Tracking cell movement

Go to the Cell Tracking tab (Fig 55). A text will appear which says that frames must be added in order to analyze images for cell tracking (Figure 56).

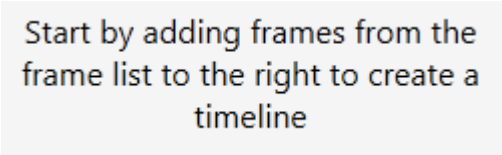

Figure 9: The Cell Tracking instruction text

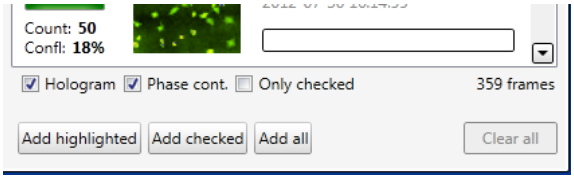

Figure 10: The Add buttons

7.1.1 Adding frames to the analysis

Highlight or check the images to be analyzed, in the Image Frame List to the right. Click the appropriate Add button (Figure 57). The Add buttons are found below the Image Frame List. The added image frames will be shown in the Source Frames window (Figure 58) below the Cell Tracking window (Figure 59).

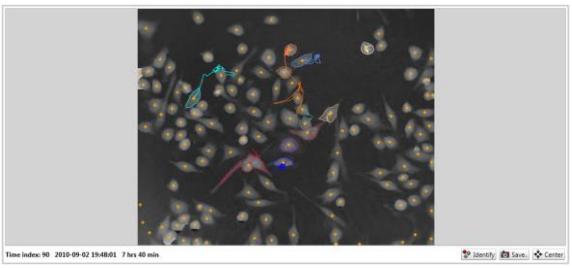

Figure 12: The Cell Tracking window

7.1.2. Adding cells

In order to follow a cell through a timelapse, the cell needs to be added. Go to the Select Mode side window (Figure 60), and activate Add Cells.

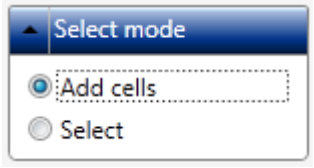

Figure 8: The Select Mode side window

In the Cell Tracking window, the center of each identified cell is marked with a small orange **+** (Figure 61). If the identification is not satisfying, go to the Identify Cells tab, and adjust the cell identification (Chapter 5).

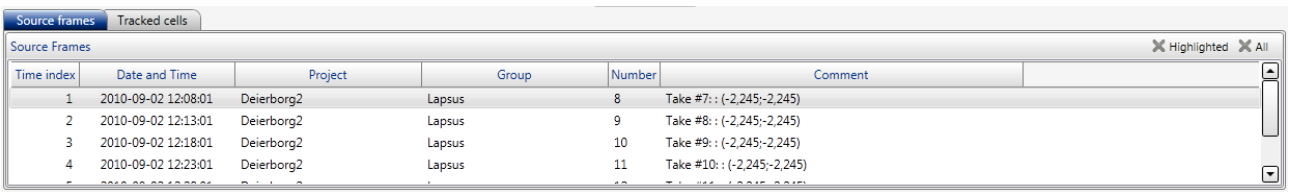

Figure 11: The Source Frames window

Click each cell to be followed (Figure 61). The cell will be followed from the frame where it is added.

Figure 61: Clicking to add a cell to the tracking results

7.1.3. Displaying the cell tracking

There is a Timeline below the Cell Tracking window (Figure 63). By moving the small gray bar, the cell movements will be displayed in the Cell Tracking window.

As the cells are followed, tracks showing their movement will be displayed (Figure 62).

The movements can also be seen in the PlotMovement tab, which is found behind the cell tracking window.

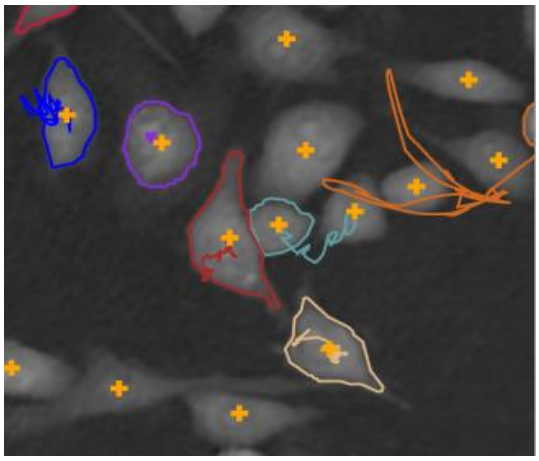

Figure 14: Tracks showing cell movements

Figure 13: Timeline for cell tracking

7.2. Adjusting the cell tracking

Sometimes the software will track the wrong cell, e.g. when cells are moving very close to each other and then separate again. This needs to be adjusted manually.

Note that the adjustments will be active from the current frame.

7.2.1. Select the cell to be adjusted

Activate Select in the Select Mode side window (Figure 60). Click on the cell to be adjusted. A new set of functions will then be available in the Change Tracking side window (Figure 64). The cell that will be adjusted is noted in the Change Tracking side window.

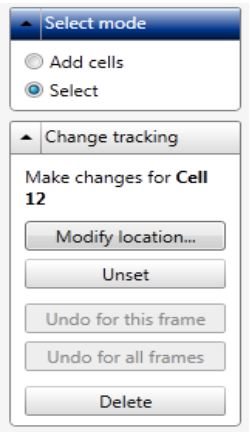

Figure 64: Changing the cell tracking

7.2.2. Switch the tracking from one cell to another

After selecting the cell to be changed, click the Modify Location button in the Change Tracking side window. Then click the cell that should actually be followed instead of the selected cell. Now, the colored border is transferred to the new cell to be followed (Figure 65).

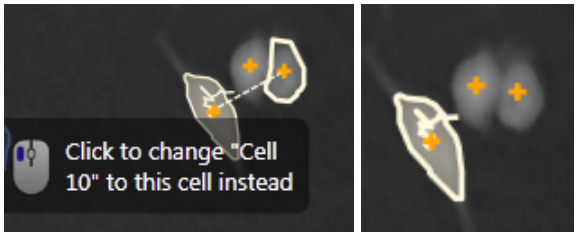

Figure 65: Transfer the tracking from one cell to another. The left image shows the selected cells, and the right image shows how the tracking is transferred.

The software will recalculate the tracking automatically from the present image frame and forward through the time lapse.

7.2.3. Discontinue a cell tracking

Sometimes a tracking needs to be discontinued, e.g. when a cell leaves the image area.

After selecting the cell to be changed, click the Unset button in the Change Tracking side window (Figure 64). The tracking will be dis-

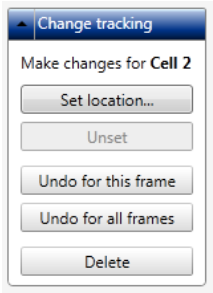

Figure 66: Set Location

continued from the present frame.

A new button, Set location, will appear in the Change Tracking window (Figure 66).

7.2.4. Continue a discontinued cell tracking

When a cell has been unset it will still be present in the Tracked Cells list, but noted as not present. In order to resume the tracking in a different frame, select the cell in the Tracked Cells list and click the Set location button (Figure 66). Then click the cell in the frame where the tracking should be resumed.

7.2.5. Undo manual changes

If the manual changes need to be undone, start by selecting a cell. Then click either the Undo for this Frame, or the Undo for all Frames button (Figure 66).

When clicking Undo for this Frame, the manual change in the current frame will be undone, and the tracking will be recalculated according to the change in settings.

When clicking Undo for all Frames, the manual changes in all frames will be removed and the tracking will be recalculated according to the change in settings.

7.3. Export the tracking results

The raw data can be exported into an xml file by clicking the Export button (Figure 67).

The image currently displayed in the cell tracking window can be exported by clicking the Save button found below the Cell Tracking window (Figure 68).

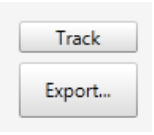

Figure 67: Track and Export buttons found to the right of the Cell Tracking window

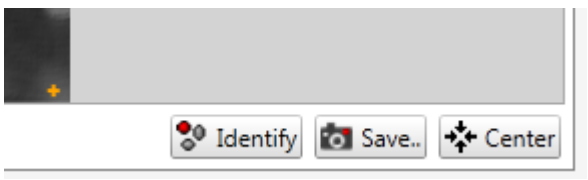

Figure 15: The Identify, Save and Center buttons found below the Cell Tracking window

Chapter 8. Export images and movies

In the Export Images tab, image frames can be edited and exported either as individual images in several standard formats or as AVI movies (Figure 69).

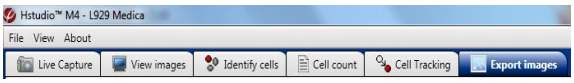

Figure 69: The Export Images tab

8.1. Add and remove image frames

Below the Image Frame list (Figure 71) there are buttons to add image frames to the Main Window. The left side windows become active only when one or several images have been added.

By clicking the Add Highlighted button (Figure 71), the data from a single image frame or from several frames can be added when the frames are highlighted in the Image Frame list. The shift key can be used to highlight several consecutive images and the ctrl key to highlight non-consecutive images.

Alternatively, the box to the left of each image can be checked and then the Add Checked button is clicked.

All image frames can be added by clicking the Add All button.

The images can also be added by clicking the images, dragging and dropping them into the Source Frames window.

Clicking the Remove buttons (Figure 70) will remove either only the highlighted or all of the added images.

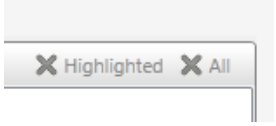

Figure 70: The Remove buttons

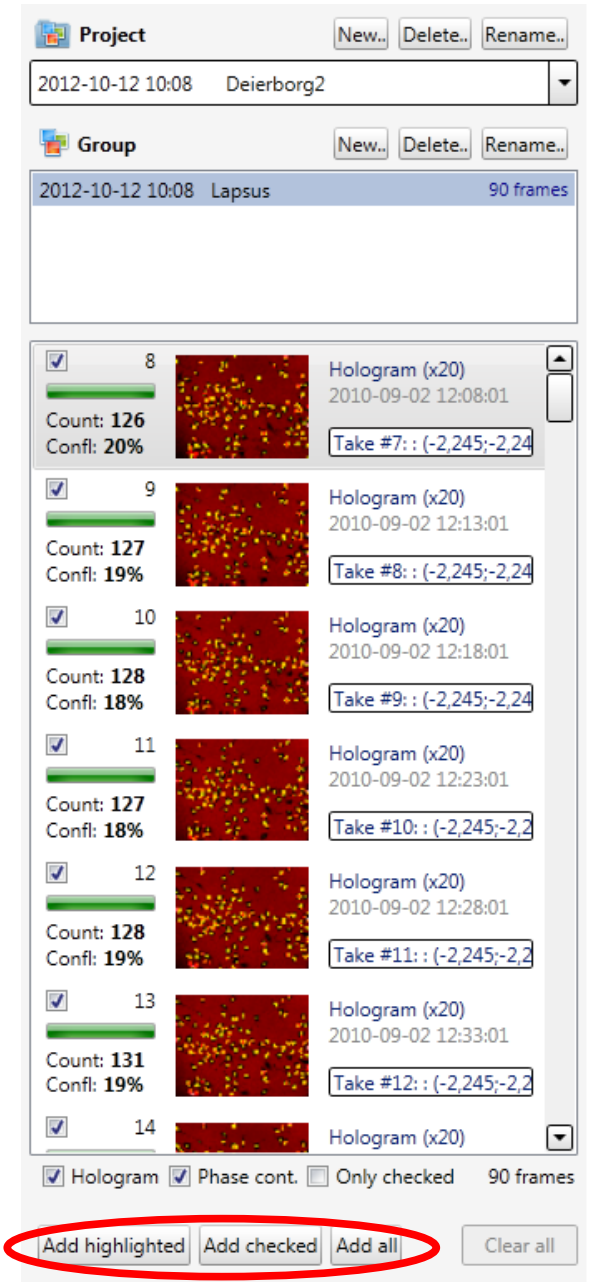

Figure 71 The Image Frame list

The Main Viewing window contains a view of the currently active added image and the Source Frames Window (below) contains thumb nails of all the added images (Figure 72).

By clicking a thumb nail, the image will *be displayed in the Main Viewing window.*

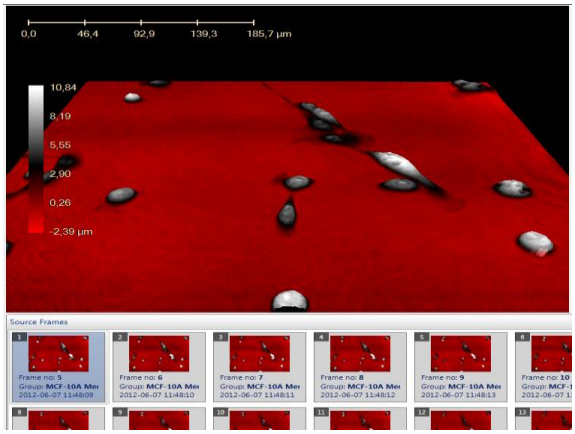

Figure 72 The Main Viewing window and the Source Frames window

8.2. Edit the images

8.2.1. Zoom, move or flip the image

The Perspective side window (Figure 73) shows how the image has been moved, flipped or zoomed.

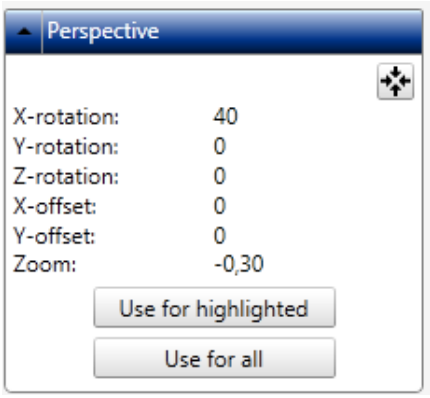

Figure 73: The Perspective side window

To zoom, left click the cell image in the Main Viewing window and then use the mouse scroll button.

To move the image to a desired location in the Main Viewing window, click, hold and drag using the left mouse button.

To flip and move the 3-D image, click, hold and drag using the right mouse button.

Click one of the Use buttons to apply the changes to the added images that are highlighted or to all added images.

8.2.2. Adjust the image display

The Viewer Options side window (Figure 74), which is found to the left of the Main Viewing window, is used to adjust the image display. Some of the options are inactive when a phase contrast image is viewed.

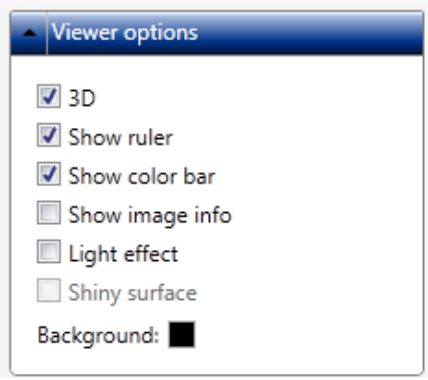

Figure 74: Viewer Options side window

Checking 3-D displays the image as a 3-dimensional representation.

Checking Show Ruler displays a horizontal scale bar representative of the distance in X and Y.

Checking Show Color Bar displays a vertical scale bar representative of the height in Z.

Checking Show Image Info displays additional information associated with the image such as (Figure 110):

First row: specifies in which project the image is located

Second row: specifies in which group the image is located

Nbr: specifies which number the image has in the group.Type: specifies if the image is holographic or phase

contrast.Date: specifies the capture date and time of the image

Checking Light Effect applies an artificial light source to the image which may sometimes render an improved image.

Checking Shiny surface applies a change in the surface image display that sometimes renders a better image. Shiny surface is only active when Light Effect is checked.

To change the Background color, left click the Background color box and select a new color (Figure 75).

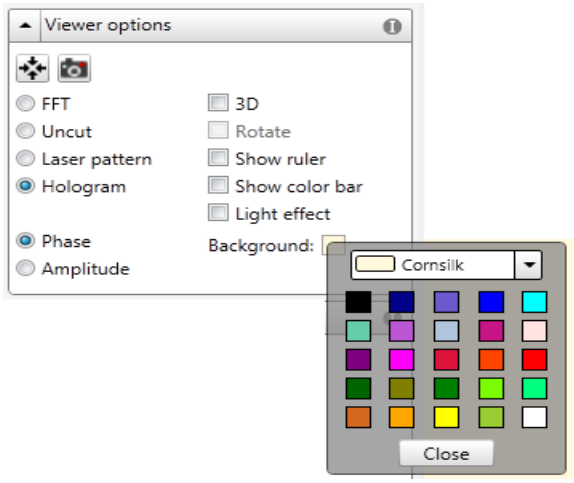

Figure 75: The Background color setting

8.2.3. Holographic image coloring

Adjust the holographic image color display and the image dynamics with the Coloring side window (Figure 76).

A set of colors that are saved together is called a colorset. A previously saved colorset can be used with the current image by making a selection in the Colorset list which is found at the top of the Coloring side window (Figure 76).

Additional functions are found in buttons and in a menu which is found at the arrow tip (Figure 77).

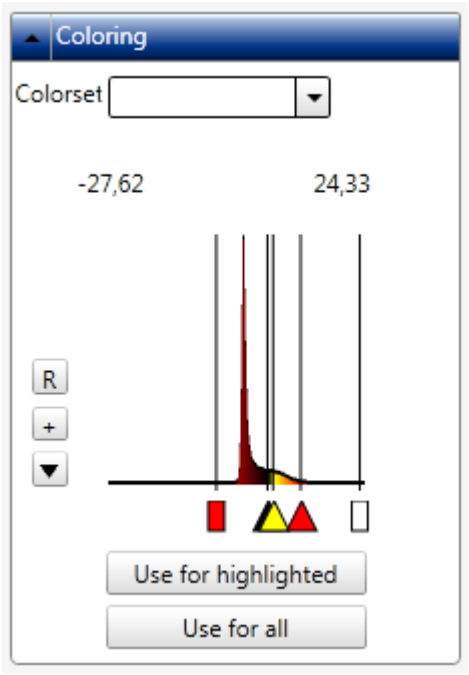

Figure 76: The Coloring side window

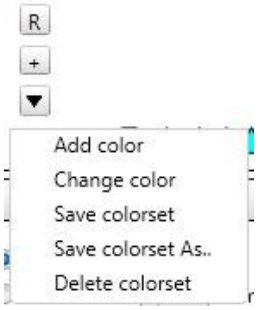

Figure 77: Additional functions found in the Coloring side window

8.3. Create an AVI movie

When the set of images look good, preview the movie by clicking the Play button (Figure 78). The number of slides per second are not shown as in the finished movie, but is rather at a set speed.

Figure 78: The Preview side window

If the preview looks good, click the Export Movie button in the Export window (Figure 79).

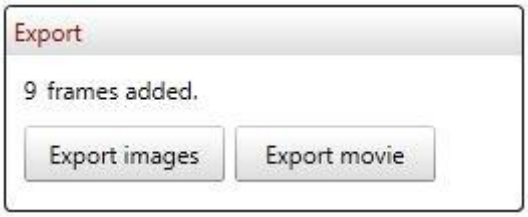

Figure 79: The Export window

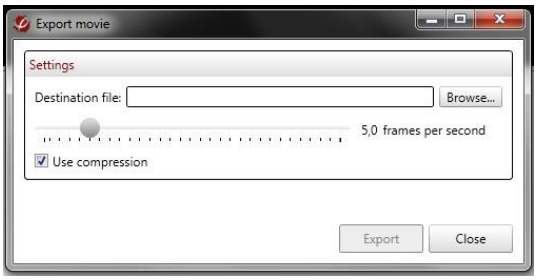

Figure 80: The Export Movie side window

Clicking the Export Movie button will open an export window (Figure 80). Select the destination folder by browsing. By moving the slide bar it is possible to set the number of frames per second that will be shown in the AVI movie.

8.4. Export images

When the image has been set up nicely and looks good, click the Export Images button to open an export window (Figure 81). Select the destination folder by browsing. Check the box to add frame comments to the file name. The Image format can be selected in the drop menu. There are five different formats to choose from: bitmap, GIF, jpg, png and TIFF.

When the box for Export Raw Images is checked, no coloring or 3-D will be displayed in the exported image.

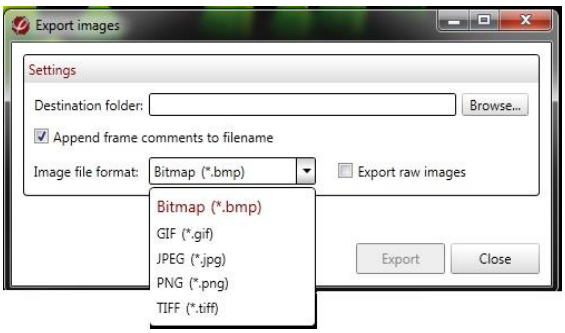

Figure 81: The Export images side window

Manual PART THREE
A list of functions

Here the functions of all tabs and side windows are presented from top to bottom and from left to right.

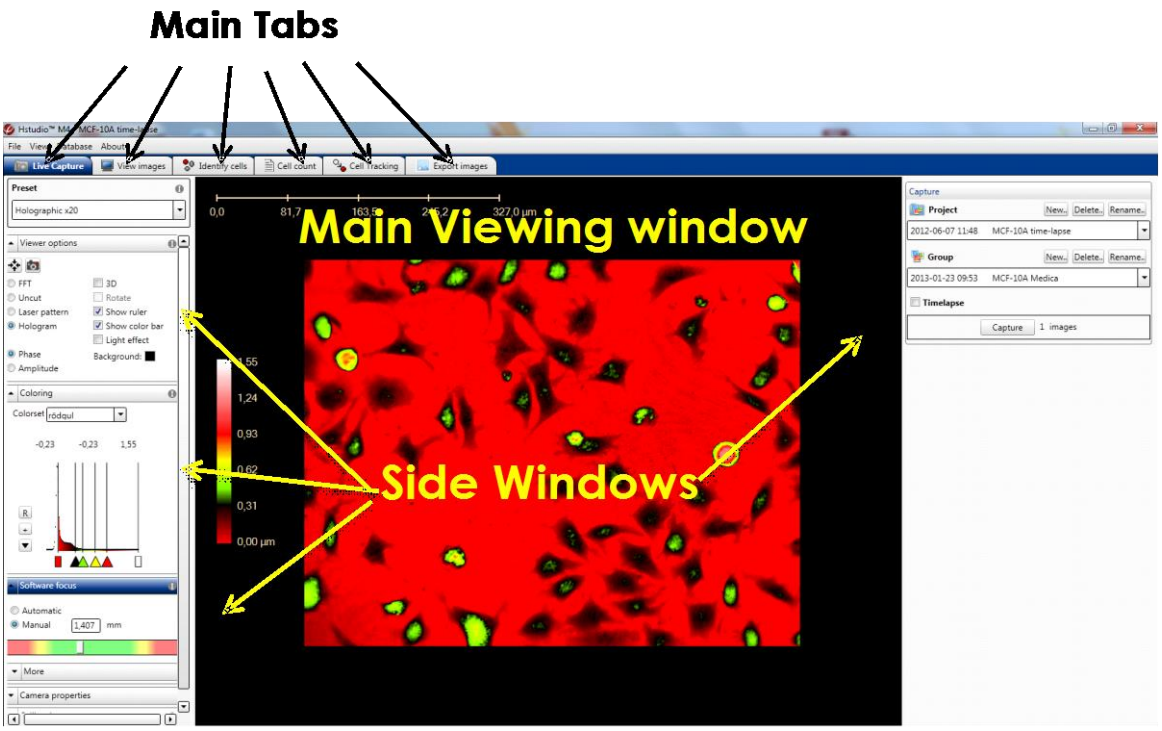

Figure 82: Overview of a main tab

HoloStudio M4 ™ is divided into six functional parts that are represented in six different tabs (Figures 82 and 83):

1. **Live Capture,** which concerns live viewing and capturing of digital hologram and phase contrast images.
2. **View Images,** which concerns viewing captured images.
3. **Identify cells,** which concerns the segmentation of an image, resulting in the outlining of the cell.
4. **Cell Count,** which concerns the analysis of the captured hologram images and display and export of the results.
5. **Cell Tracking,** which concerns the tracking of single cells through a series of captured frames.
6. **Export Images,** which concerns the visualization and export of images and movies.

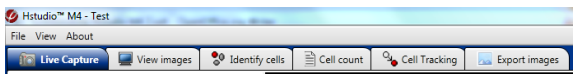

Figure 83: The main tabs of HoloStudio M4

The Main Viewing window

The Main Viewing window (Figure 82) shows the live image in the Live Capture tab and saved images when the other tabs are active.

The Side windows

Most functions are found in the side windows for each tab. If the side windows are collapsed they can be expanded by clicking the black arrow tip found in every side window header.

Additional functions or parameters are found in the More menus in some of the side windows. These functions are usually not needed for the user but rather for the service engineers.

All changes performed on the displayed images are temporary until the user chooses Save or Apply to. Unless the image is deleted, the raw data will always remain intact as any changes the user makes, only concern how the results are displayed.

Information concerning the different side windows can be found by clicking the Information buttons for each side window (Figure 84).

Figure 84: Information button

Chapter 9. Live Capture

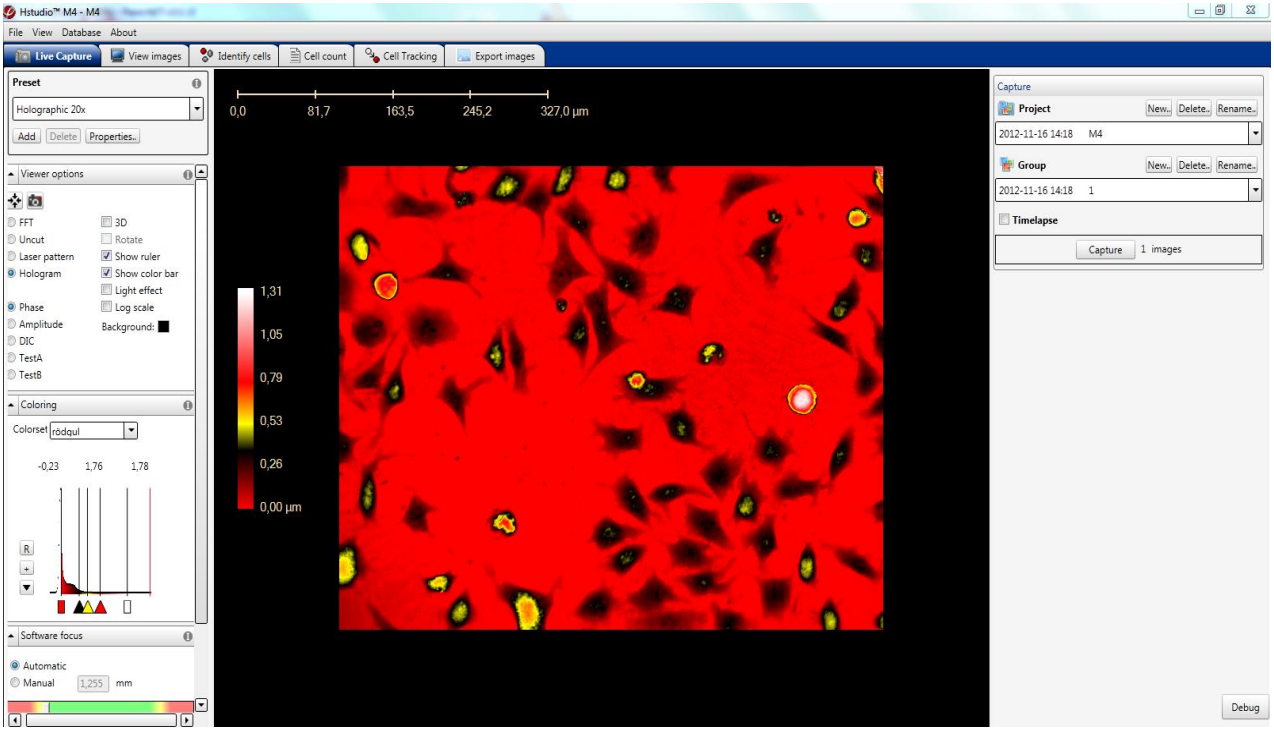

Figure 85: The Live Capture tab

In the Live Capture tab, live holographic images can be viewed and captured (Figure 85).

Note that if the software is started without a connected HoloMonitor M4, the Live Capture tab will be inactive.

Collapsed Side windows can be expanded by clicking the black arrow tip found in every side window header.

Here follows descriptions of the functions that are found in the Side windows for the Live Capture tab.

9.1 Presets

In the Presets side window, the objective is selected (Figure 86). The HoloMonitor M4 has a 20x objective.

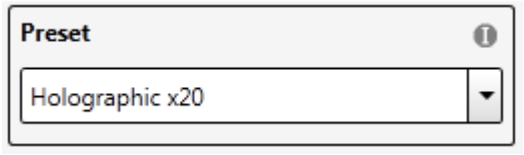

Figure 86: The Preset side window

9.2. Viewer Options

The Viewer Options side window (Figure 87) on the left hand side of the Main Viewing window is used to change how the live image is displayed.

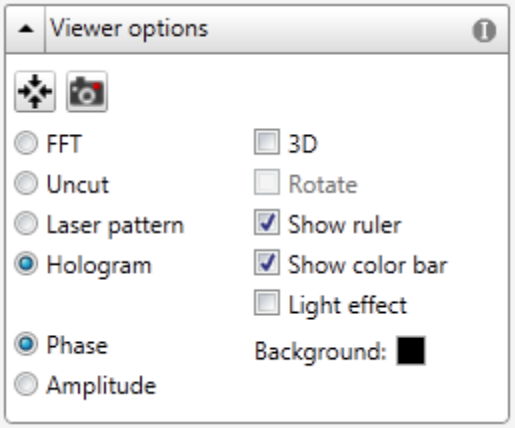

Figure 87: The Viewer Options side window

By clicking the Center button (Figure 88) the image will be centered in the Main Viewing window.

Figure 88: The Center button

By clicking the Camera button (Figure 89) the image currently displayed in the Main Viewing window will be saved as an image.

Figure 89: The Camera button

The following functions are available in the Viewer options side window. They are activated by checking the boxes. Most boxes can be checked in parallel, enabling the user to combine functions.

Checking FFT displays the Fast Fourier Transform which represents the frequency domains.

Checking Uncut displays the image as it is first reconstructed.

Checking Laser Pattern displays the original interference pattern resulting from the merging of the object and reference laser beams.

Checking Hologram displays the reconstructed image which is based on the laser pattern. The hologram can be displayed showing either the phase or amplitude information of the light wave.

Checking Phase displays the light wave phase information in the hologram.

Checking Amplitude displays the light wave amplitude information in the hologram.

Checking 3-D displays the holographic data as a 3-dimensional representation.

Checking Rotate auto-rotates the image.

Checking Show Ruler displays a horizontal scale bar representative of the distance in X and Y.

Checking Show Color Bar displays a vertical scale bar representative of the height in Z.

Checking Light Effect applies an artificial light source to the image which may sometimes render an improved image.

Checking Shiny Surface applies a change in the surface image display that sometimes renders a better image. Shiny surface is only active when Light Effect is checked.

To change the Background color in the Main Viewing window, left click the Background color box and select a new color (Figure 90).

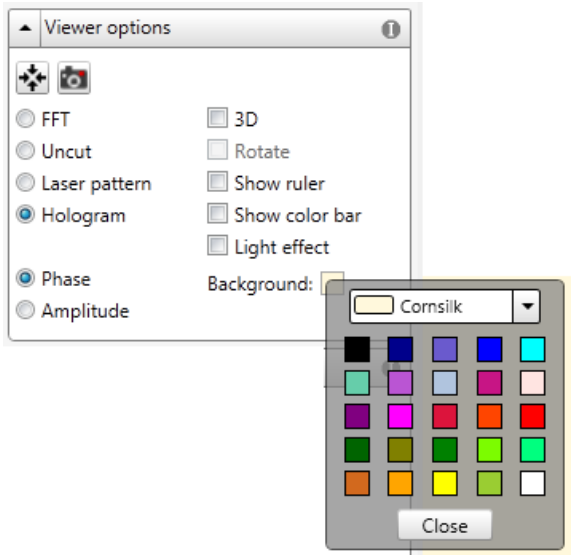

Figure 90: The Background color setting

making a selection in the Colorset list which is found at the top of the Coloring side window (Figure 91).

Additional functions are found as buttons and at the black arrow tip button (Figure 92)

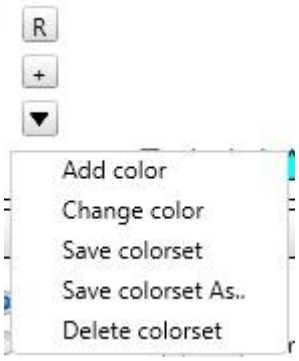

Figure 92: Additional functions found in the Coloring side window

9.3. Coloring

The Coloring side window (Figure 91) is used to adjust the holographic image color display in the Main Viewing window and to adjust the image to optimally display the image dynamics.

A set of colors that are saved together is called a colorset. A previously saved colorset can be used with the current image by

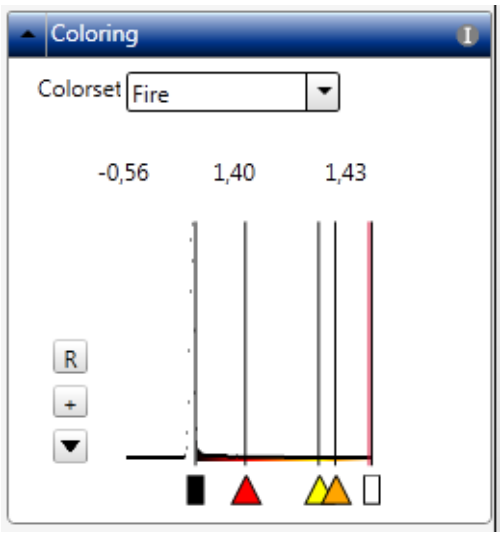

Figure 91: The Coloring side window

By left clicking the R-button (Figure 92) the coloring in the image is rescaled to better utilize the optimal dynamic range of the image. This button needs to be operated every time the image coloring is off.

To add a new color to the colorset, left click the plus button (Figure 92) and select a new color. A colored triangle representing the new color will appear beneath the histogram (Figure 91). Alternatively, right click the x-axis at the position where a new color is wanted and select Add Color from the menu that appears.

Change the color by using the right mouse button to click on a colored triangle beneath the histogram and select a new color, alternatively left click the arrow button and select Change Color (Figures 91 and 92).

To change the color span, left click and move the desired colored triangle beneath the histogram using the cursor (Figure 91).

To save a new colorset with the current color settings, left click the arrow button and choose Save As (Figure 92).

To save the current color settings to a previously saved colorset, left click the arrow button and choose Save (Figure 92). Note that this will overwrite the settings previously saved to this colorset.

To delete a colorset, select it in the Colorset list by left clicking it. Then left click the arrow button and select Delete Palette (Figure 92).

9.4. Software Focus

9.4.1. Automatic focus

The software focus is calculated either automatically or manually. Automatic software focusing (Figure 93) mostly results in well focused images.

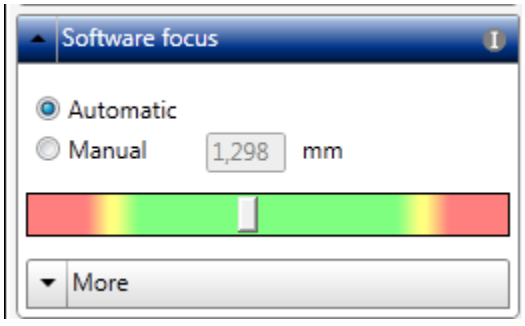

Figure 93: The Software Focus side window set to Automatic

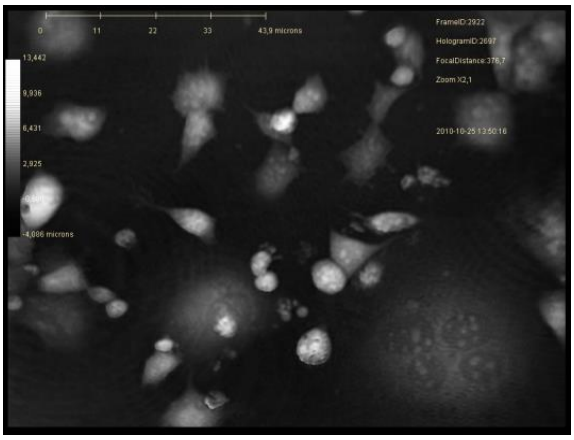

Figure 94: A holographic phase image that is in

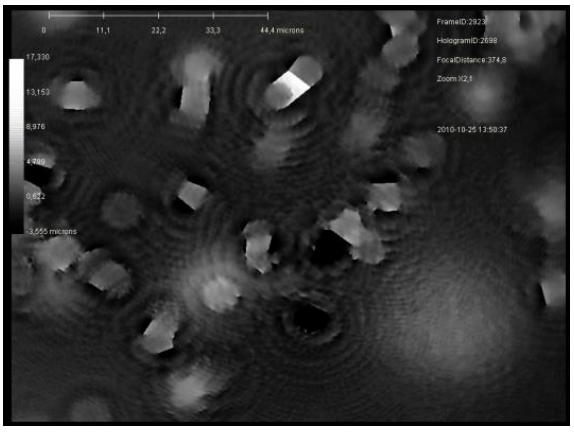

Figure 95: A holographic phase image that is out of

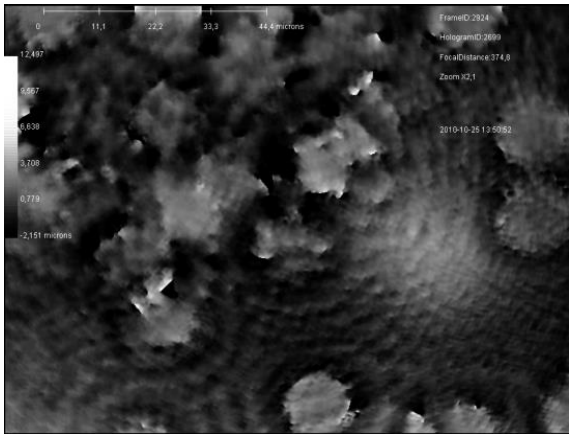

Figure 96: A holographic phase image that is totally out of focus

When Automatic is selected, the computer will calculate the optimal focus. Some cell samples are more demanding and need to be focused manually. Figures 94, 95 and 96 show holographic images that are in focus, out of focus and completely out of focus.

Adjust the focus by adding or removing adapter-plates to the sample stage.

9.4.2. Manual focus

When activating manual software focusing (Figure 97), the user will have to set the software focus distance. The text box next to the Manual button shows the software focus distance in mm. The distance can be changed either by entering a value manually or by using the slide bar beneath the text box.

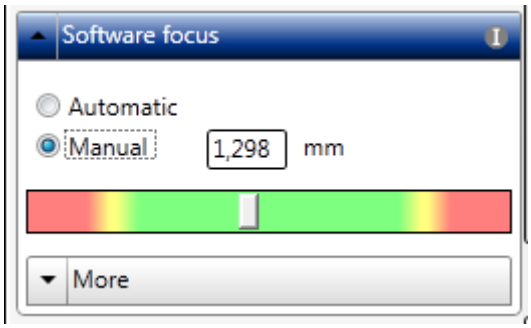

Figure 97: The Software Focus side window set to Manual

9.4.3. More

Under the More list in the Software Focus side window (Figure 98), further parameters can be found. These parameters are mainly useful for service personnel. These parameters are set during the installation of the software.

Relative center X and Y, control the cropping of the Amplitude FR Image and of the final

Relative center X is the value for the relative center of the image in the X dimension.

Relative center Y is the value for the relative center of the image in the Y dimension.

Focal length allows the software to calculate the size of the image.

hologram.

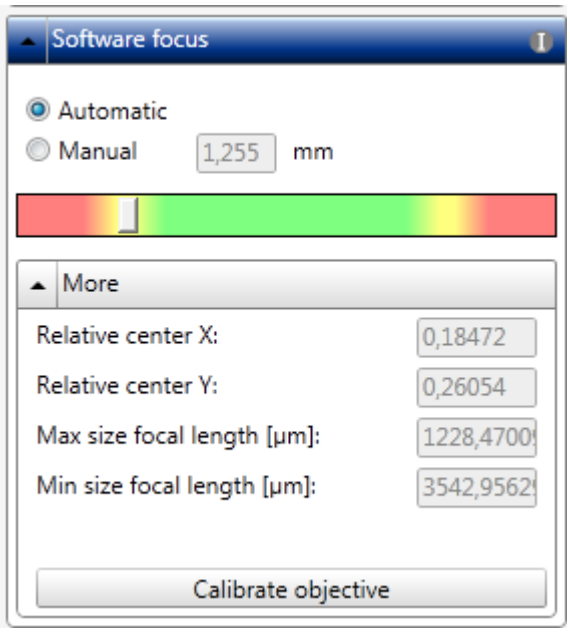

Figure 98: The More list of the Software Focus side window

9.4.4. Calibrate Objective

Calibrate Objective is used to adjust image calculations to the objective position. The sample must be removed when the calibration is performed.

9.5. Camera Properties

The Exposure Time and the Gain of the hologram camera are usually set to Auto

Exposure. When Auto Exposure in the Camera Properties side window (Figure 99) is unchecked the exposure time and the gain can be set manually either by entering values in the text boxes or by using the slide bars.

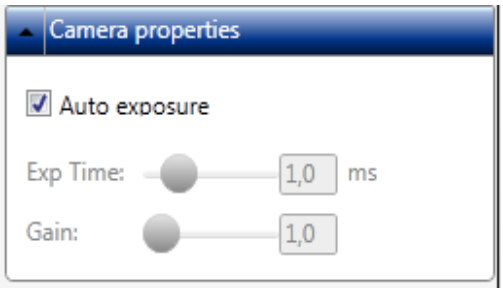

Figure 99: The Camera Properties side window

9.6. Calibration

In the Calibration side window (Figure 100), the background can be calibrated by clicking the corresponding buttons.

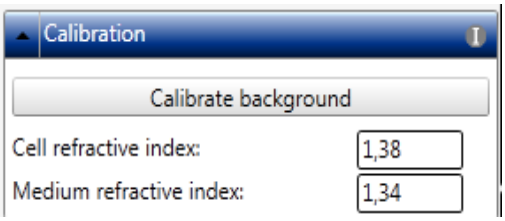

Figure 100: The Calibration side window

Background calibration is performed to achieve a higher image quality. It subtracts the background noise from the captured image. The sample must be removed when the calibration is performed.

It is necessary to fill in the correct cell and medium refractive indexes in order for the algorithms to reconstruct the cell image correctly.

> Cell refractive index is the refractive index of the cultured cells. A common cell refractive index is 1.38.
>
> Medium refractive index is the refractive index of the cell culture medium used. A common cell culture medium refractive index is 1.34.

9.7. Capture

The Capture side window (Figure 101) is located to the right of the Main Viewing window and is used to set the parameters for image capture.

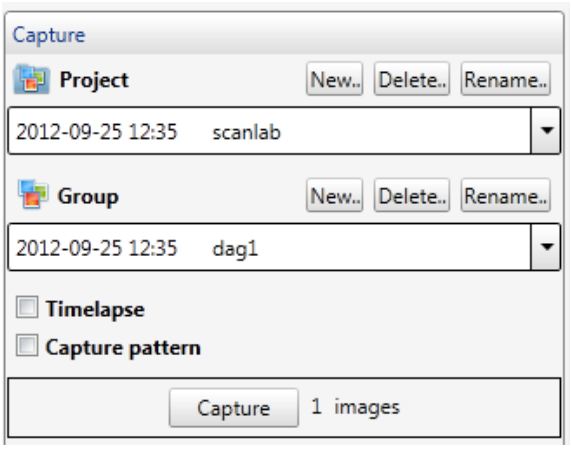

Figure 17: The Capture side window

In the upper part of the Capture side window a Project and Group, where the captured images will be saved, can be selected or created. Unless a Project and a Group are selected, the Capture button remains inactive and images cannot be captured.

9.7.1. Capture a single image

Clicking the active Capture button, result in one captured image.

9.7.2. Capture a timelapse

To enable slow events to be recorded and studied, a movie can be created from images captured at intervals, *i.e*. a timelapse movie.

After checking Timelapse in the Capture side window (Figure 102) the total time of the timelapse should be entered in the corresponding text box. Then select the desired time units (seconds, minutes or hours).

Thereafter the interval with which the images should be captured must be entered. The shortest possible interval is given beside the interval box.

The time-controlled capture starts when the Capture button is clicked. The total number of captures is given beside the Capture button.

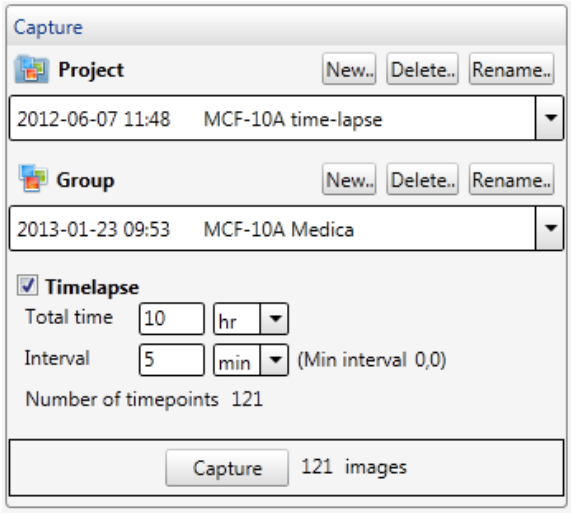

Figure 102: The Timelapse function in the Capture side window

Chapter 10. View Images

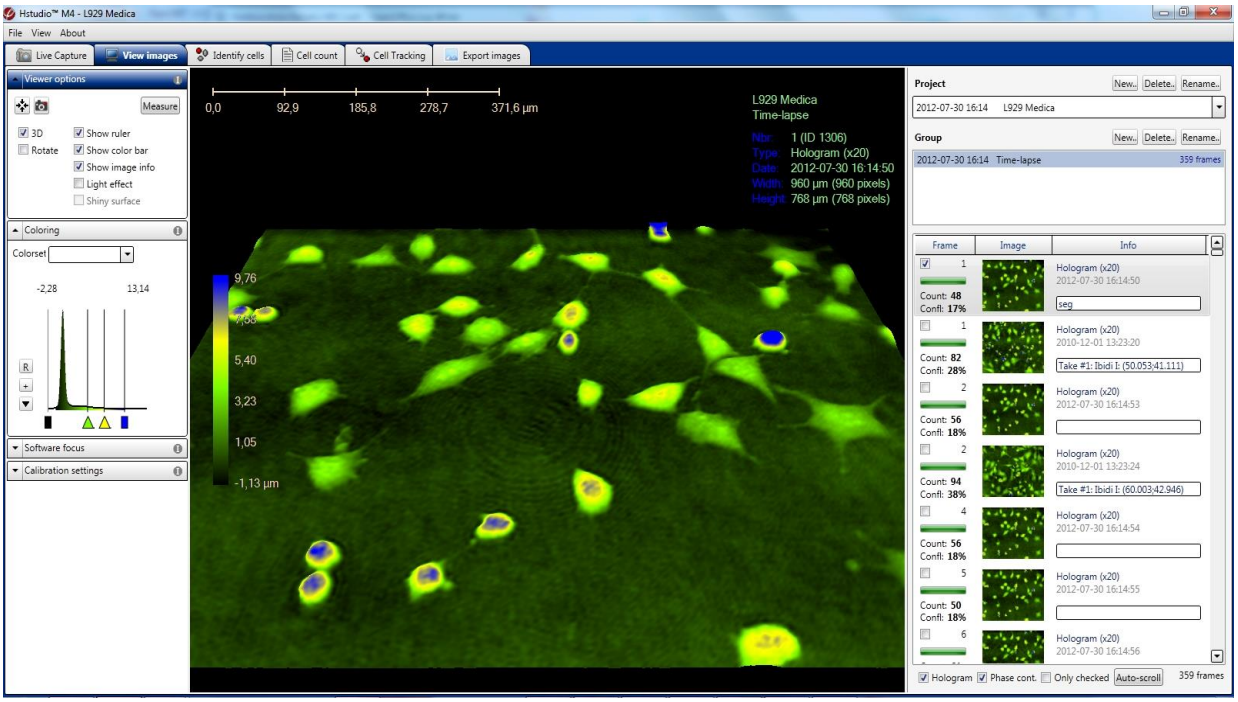

Figure 103: The View Images tab

In the View Images tab (Figure 103), captured images can be viewed and artificially colored and their software focus can be recalculated. This tab also contains a tool where cells can be measured manually.

The following functions are found in the side windows of the View Images tab. If the side windows are collapsed they can be expanded by clicking the black arrow tip found in every side window header.

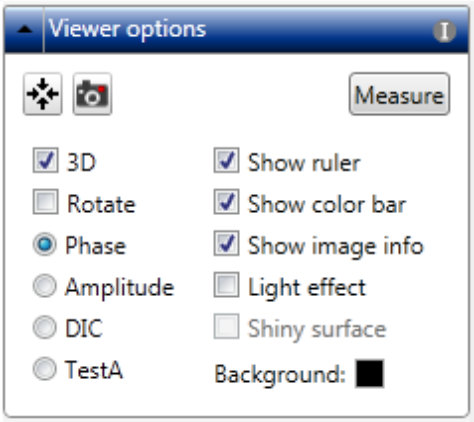

Figure 104: The Viewer Options side window

10.1. Viewer Options

The Viewer Options side window (Figure 104) on the left hand side of the Main Viewing window is used to change how the image is displayed.

10.1.1. Buttons in Viewer Options

When clicking the Center button (Figure 105) in the Viewer Options side window, the image will be centered in the Main Viewing window.

Figure 105: The Center button

Figure 106: The Snapshot button

When clicking the Snapshot button (Figure 106) in the Viewer Options side window, an image of the current view will be saved.

10.1.2. The Measure Button

When clicking the Measure button (Figure 107) in the Viewer Options side window, the user can make manual measures of objects in the current image.

When the measuring function is activated, the measurement is started by left clicking anywhere in the image. The measurement is finished by left clicking at a new point in the image.

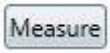

Figure 107: The Measure button

The measured distance is shown with a blue bar (Figure 108). A window displaying the results, appear to the bottom right of the main viewing window. The results include a profile of the measured object. The maximum thickness of the object is shown as red text by the dashed line.

The data are saved as an image when the Snapshot button is clicked (Figure 106).

It is possible to move the image in the Main Viewing window while the measuring function is activated. The image is moved by clicking and dragging.

When the Measure button is clicked again, the measuring function is deactivated.

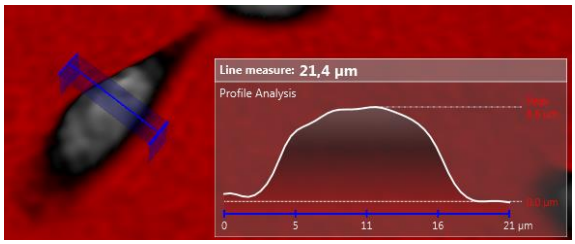

Figure 108: The blue measuring bar and the results window

10.1.3. The display options in the Viewer Options window

The different display functions in the Viewer Options side window (Figure 104) can be activated by checking the boxes. Most boxes can be checked in parallel to combine functions.

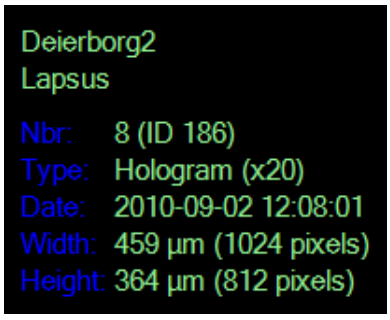

Figure 109: Image information for holographic images

Checking 3-D displays the holographic data as a 3-dimensional representation.

Checking Rotate auto-rotates the image.

Checking Show Ruler displays a horizontal scale bar representative of the distance in X and Y.

Checking Show Color Bar displays a vertical scale bar representative of the height in Z.

Checking Show Image Info displays additional information associated with the image such as (Figure 109):

- Project, specifying in which project the image is located.
- Group, specifying in which group the image is located.
- Nbr, specifying which number the image has in the group.
- Type, specifying if the image is holographic or phase contrast.
- Date, specifying the capture date and time of the image.
- Width, specifying the image width in µm and pixels.
- Height, specifying the image height in µm and pixels.

Checking Light Effect applies an artificial light source to the image which may sometimes render an improved image.

Checking Shiny Surface applies a change in the surface image display that sometimes renders a better image. Shiny Surface is only active when Light Effect is checked.

10.2. Coloring

The Coloring side window (Figure 110) allows the user to adjust the image colors displayed in the Main Viewing window and to adjust the image to optimally display the image dynamics.

A set of colors that are saved together is called a colorset. A previously saved colorset can be used with the current image by making a selection in the Colorset list which is found at the top of the Coloring side window (Figure 110).

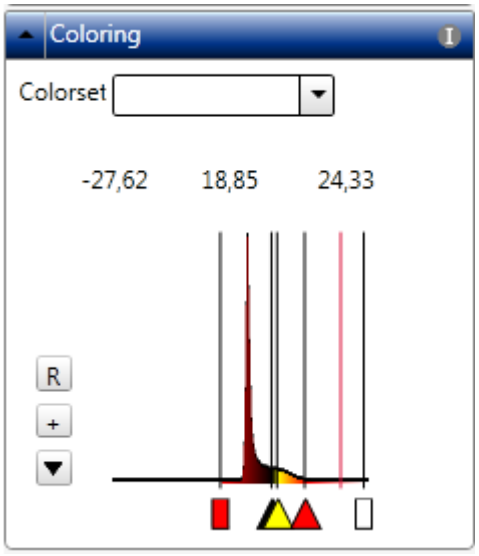

Figure 110: The Coloring side window

When left clicking the R-button (Figure 110), the coloring in the image is rescaled to better utilize the optimal dynamic range of the image. This button needs to be operated every time the image coloring is off.

To add a new color to a colorset, left click the plus button (Figure 111) and select a new color. A colored triangle representing the new color will appear beneath the histogram (Figure 110). Alternatively, right click the x-axis at the position where a new color is wanted and select Add Color from the menu that appears.

Change the color by using the right mouse button to click on a colored triangle beneath the histogram and select a new color, alternatively left click the arrow button and select Change Color (Figures 110 and 111).

To change the color span, left click and move the desired colored triangle beneath the histogram using the cursor (Figure 110).

To save a new colorset with the current color settings, left click the arrow button and choose Save As (Figure 111).

To save the current color settings to an already existing colorset, left click the arrow button and click save (Figure 111). Note that this will overwrite the settings previously saved to this colorset.

To delete a colorset, select it in the Colorset list. Then left click the arrow button (Figure 111) and click Delete palette.

To save a colorset to an image, left click the arrow button (Figure 111) and choose Apply To, followed by Frame. The current colorset will be applied to the currently viewed frame and saved.

To save the current colorset to several images, check the box of each of the desired images in the Image Presentation side window (Figure 112). Then left click the arrow button in the Coloring side window (Figure 111). Choose Apply To, followed by left clicking Checked Frames. The current colorset will be applied to the checked image frames and saved.

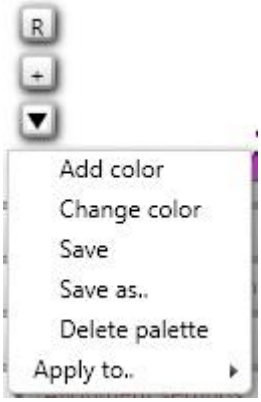

Figure 111: Additional functions found in the Coloring side window

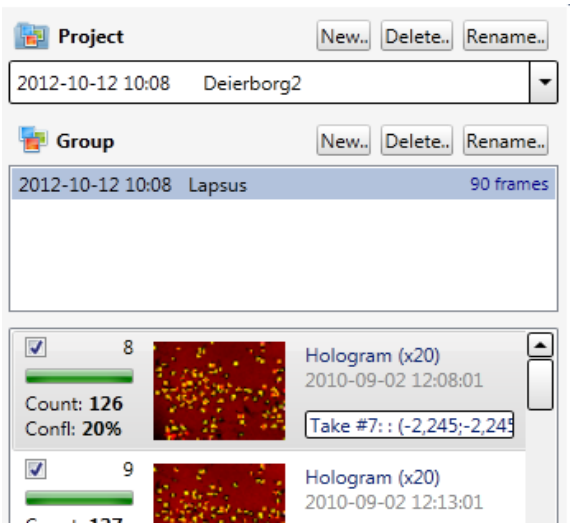

Figure 18: The Image Frame list

10.3. Software Focus

Automatic software focusing mostly results in well focused images. Some cell samples are more demanding and need to be focused manually. The Software Focus side window (Figure 113) allows the user to choose whether the software focus is calculated automatically or manually even after the image is captured.

Figures 114, 115 and 116 show holographic images that are in focus, out of focus and totally out of focus.

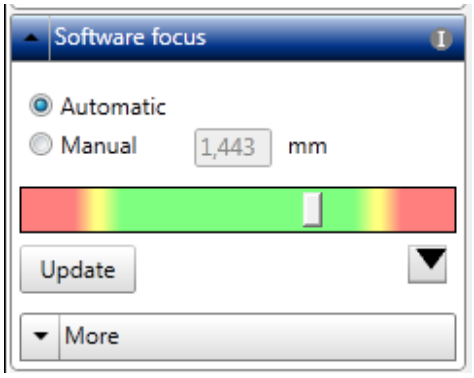

Figure 113: The Software Focus side window set to Automatic

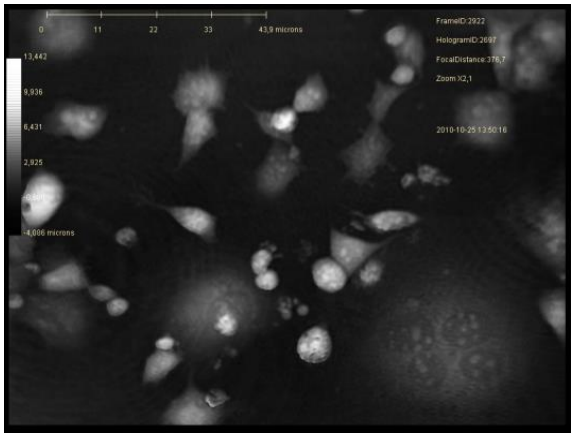

Figure 19: A holographic phase image that is in focus

10.3.1. Automatic

Using automatic calculation settings, the computer will calculate the optimal focus. If an image is not correctly focused, try to click the Update button. The computer will then recalculate the software focus.

To save a focus calculation to the current image, right click the arrow button, select apply to and then frame.

To save a focus calculation to several frames, check the boxes of each image in the image presentation list. Then right click the arrow button, select Apply To and then Checked Frames.

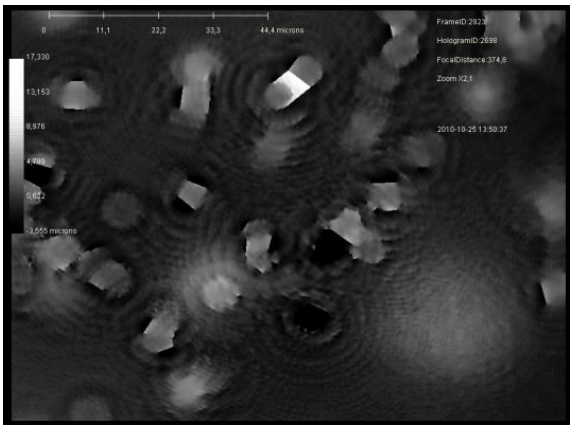

Figure 115: A holographic phase image that is out of focus

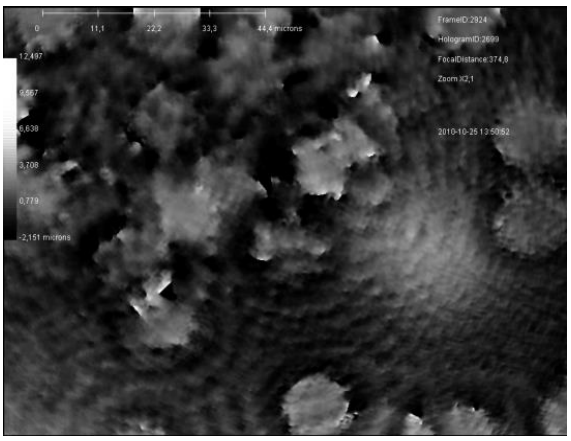

Figure 116: A holographic phase image that is totally out of focus

10.3.2. Manual

When activating manual software focusing, the user will have to adjust the focus distance. The text box next to the Manual button (Figure 117) shows the software focus distance in mm. The distance can be changed either by entering a value manually or by using the slide bar beneath the text box.

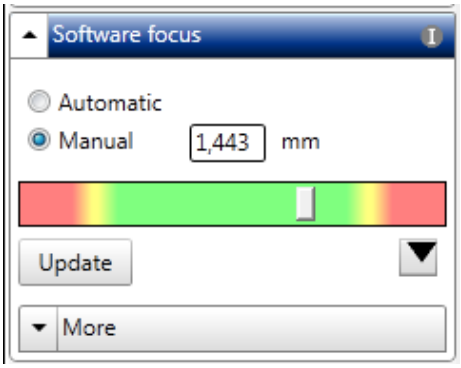

Figure 117: The Software Focus side window

To save a focus calculation to the current image, right click the arrow button, select

apply to and then frame.
To save a focus calculation to several frames, check the boxes of each image in the image presentation list. Then right click the arrow button, select Apply To and then Checked Frames.

10.3.3. More

Under the More list, in the Calculation settings side window (Figure 118), further functions can be found. These functions are mainly useful for service personnel.

The Relative center X and Y are used to control the cropping of the Amplitude FR Image and of the final Hologram.
Changes to the calculation settings can be saved with the Save button (Figure 118).

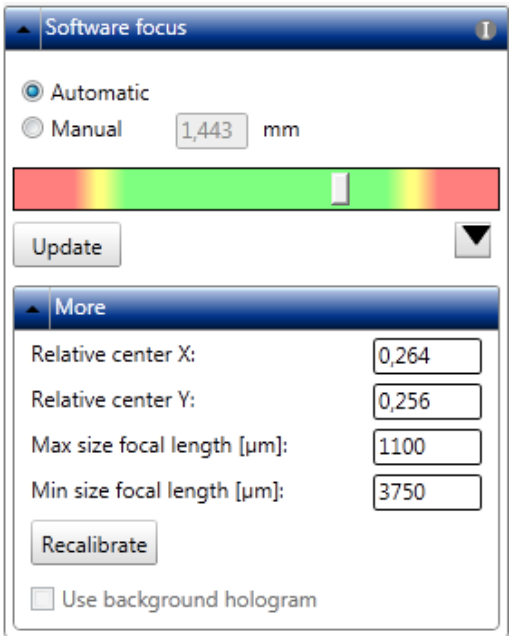

Figure 118: The More list in the Software focus side window

Relative center X is the value for the relative center of the image in the X dimension.

Relative center Y is the value for the relative center of the image in the Y dimension.

Focal length is used to calculate the size of the image.

Clicking the Recalibrate button (Figure 118) will result in a re-centered image with an adjusted focal distance. It is used for images captured with a HoloMonitor which is not properly calibrated.

10.4. Calibration Settings

In the Calibration Settings side window (Figure 119) the cell and medium refractive indexes can be changed.

Cell refractive index is the refractive index of the cultured cells. An average refractive index for cells is usually 1.38.

Medium refractive index is the refractive index of the cell culture medium used. An average refractive index for cell culture medium is usually 1.34.

By clicking the arrow button (Figure 119) changes in the refractive indexes can be applied and saved to either the current frame or to frames that are checked in the Image Frame list (Figure 120).

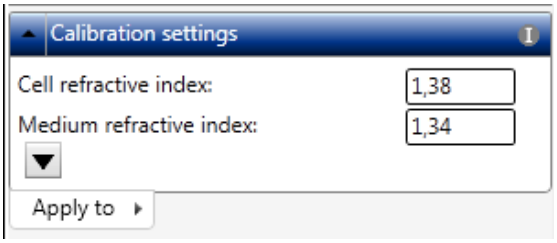

Figure 119: The Calibration settings side window

10.5. Image Presentation

To the right of the Main viewing window, the images in the currently selected project and group are presented in the Image Frame list (Figure 120).

At the top of the Image Frame list, first the Project and then the Group is selected. The image that is highlighted will be shown in the Main Viewing Window.

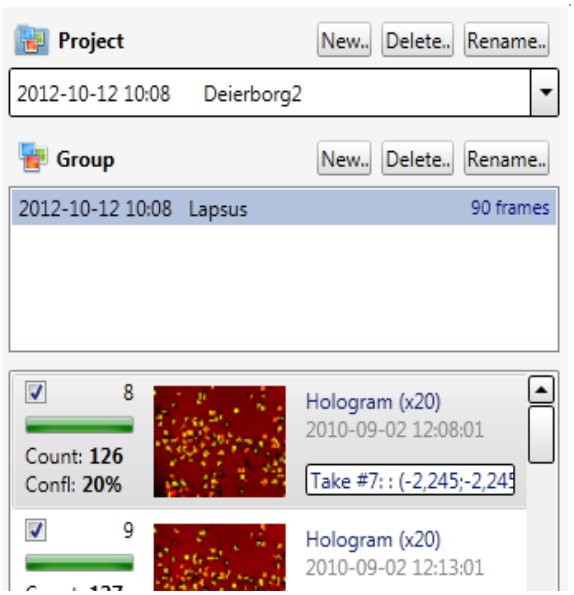

Figure 120: The Image Frame list

10.5.1. Image information

The image number is given to the left of each image thumbnail (Figure 120).

A filling green bar to the left of the image thumbnail indicates that a process is ongoing. A filled green bar indicates that no process is ongoing.

When an image is captured, cells are identified automatically. The number of cells in the frame, as well as the confluency, are given to the left of the image thumbnail.

To the right of each image thumbnail the date and time of image capture are given.

The take number and the well name of multi well vessels and the stage objective coordinates are given to the right of each thumbnail (Figure 121). In that same space the user can fill in information manually. The take number is the number of each time point in a timelapse sequence. If a timelapse is combined with a capture pattern, the images captured at the same timepoint will have the same take number.

Figure 121: Take number information

10.5.2. Check and Delete

By clicking the right mouse button while hovering over an image thumbnail, a menu is accessed containing Check, Delete and Export (Figure 122).

When Check (Figure 122) is clicked, a selection of functions become available with which it is possible to check the boxes to the left of each image thumbnail. Either only the highlighted frames can be checked or all frames. Alternatively, the boxes can be checked manually by clicking the boxes with the left mouse button.

When Uncheck (Figure 122) is clicked, the boxes to the left of each image thumbnail can be unchecked. Either only the highlighted frames can be unchecked or all frames. Alternatively, the boxes can be unchecked manually by clicking the checked boxes with the left mouse button.

The shift key can be used to highlight several consecutive images and the ctrl key to highlight non-consecutive images.

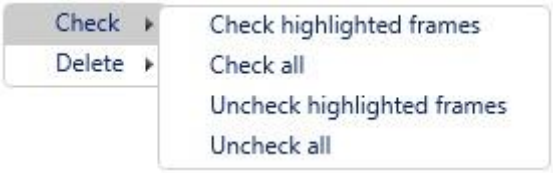

Figure 122: The Check menu

When Delete (Figure 123) is clicked, one or more highlighted or checked image frames can be deleted.

Figure 123: The Delete menu

10.5.3. Image display

Below the Image Frame list there are image display functions (Figure 124). Checking or unchecking the boxes, determines which images are displayed in the Image Frame list (Hologram and/or Phase Contrast and/or Only Checked).

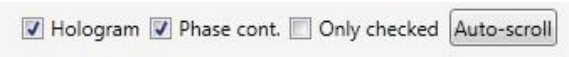

Figure 124: Image display functions

When the box for Only Checked is checked, only checked frames will be displayed.

When the Auto Scroll button (Figure 124) is clicked, the images displayed in the Image Frame list will be displayed in a sequence similar to a movie.

Chapter 11. Identify Cells

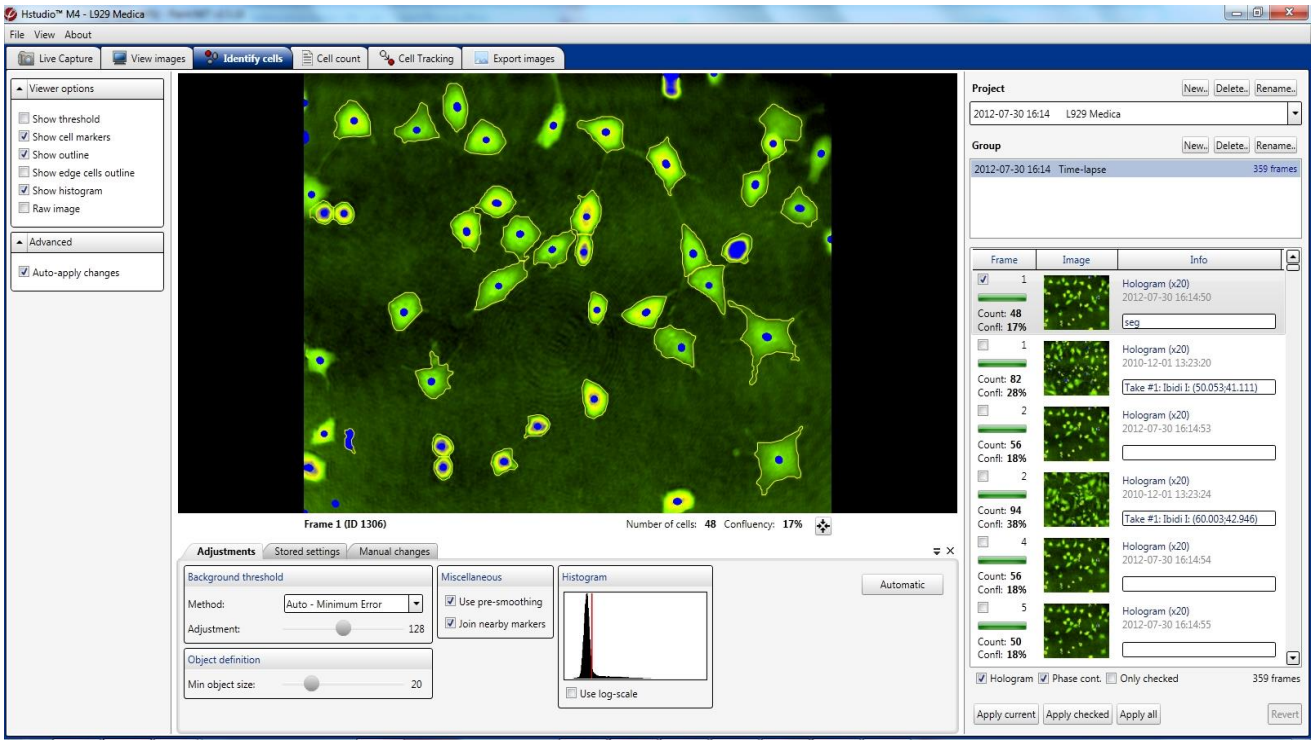

Figure 125: The Identify Cells tab

The base for all holographic image analysis is the segmentation step. In the Identify Cells tab (Figure 125), the threshold between background and cell can be adjusted and each identified cell is outlined. Only thereafter the program can determine the individual cell properties using the images.

The following functions are found in the Main Viewing window and the side windows for the Identify Cells tab. If the side windows are collapsed they can be expanded by clicking the black arrow tip found in every side window header.

11.1. Viewer Options

In the Viewer Options side window (Figure 126), on the left hand side of the Main Viewing window, it is possible to change how the image is displayed. The different display functions are shown and can be activated by checking the boxes. The boxes can be checked in parallel to combine functions.

11.2. Advanced

Using Auto-apply Changes (Figure 126) allows the user to implement all changes immediately.

Figure 126: The Viewer Options side window

Checking Show Threshold displays a red coloring that distinguishes cells from the background.

Checking Show Cell Markers displays a blue coloring that indicates the densest part of the cell.

Checking Show Outline displays a yellow coloring that indicates the border of the cells.

Checking Show Edge Cells Outline displays a yellow coloring that indicates the border of the cells touching the image edges.

Checking Show Histogram displays a histogram that shows the intensity in the image. The histogram is found below the Main Viewing window.

Checking Raw Image causes the image to be displayed as the unadulterated gray scale image.

11.3. Adjustments

Below the Main Viewing window, tabs for Adjustments, Stored Settings and Manual changes are found (Figure 127).

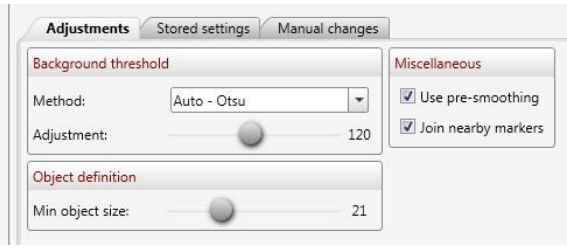

Figure 127 Adjustments, Stored Settings and Manual Changes tabs

The Adjustments tab enables the user to adjust what is counted as cell and what is counted as background. Also the size of the objects counted as cells, can be adjusted.

11.3.1. Background Threshold

The background threshold setting determines what is cells and what is background in the image. The software will automatically make threshold settings according to the threshold settings method that is currently set.

There are several different threshold setting methods in the Method list (Figure 128). The different methods to calculate the threshold settings will result in slightly different cell identifications. As the best threshold setting method is cell line and cell density dependent, it is advisable to try out which method that works best for every type of cell sample.

Manual allows the user to set the global threshold level using the slider.

Minimum error automatically sets the global threshold level using the minimum error histogram-based threshold method.

Otsu automatically sets the global threshold level using the Otsu method.

Otsu in blocks: the image is split into blocks which are thresholded separately using Otsu method. This is a form of adaptive threshold.

Adaptive mean is an adaptive thresholding method using a mean filter.

Adaptive gaussian is an adaptive thresholding method using a gaussian filter.

Double otsu: double thresholding is a method where both a wide and a narrow threshold mask is used. The narrow image is morphologically reconstructed under the wide image. The final image is used as threshold mask. The result is a cleaner threshold mask. The Double Otsu uses double thresholding with Otsu global threshold as mid-level threshold.

Double adaptive mean: same as Double Otsu but with two adaptive mean threshold masks.

Double adaptive gaussian: same as Double Otsu but with two adaptive gaussian threshold masks.

The selected method can be adjusted with the Adjustment slide bar (Figure 128) which is found below the Methods list. Different types of cells may be more precisely identified with different methods for background threshold calculation.

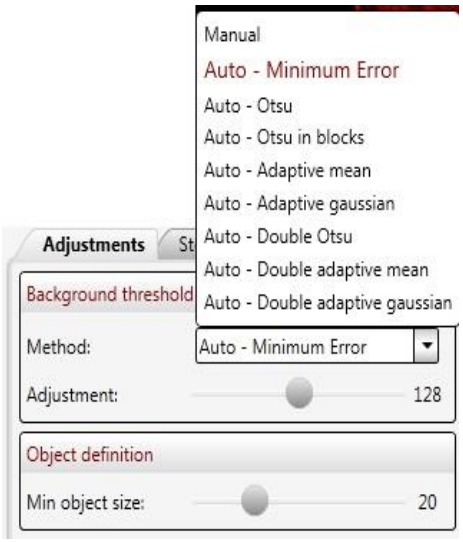

Figure 128: The different segmentation methods available in the Methods list

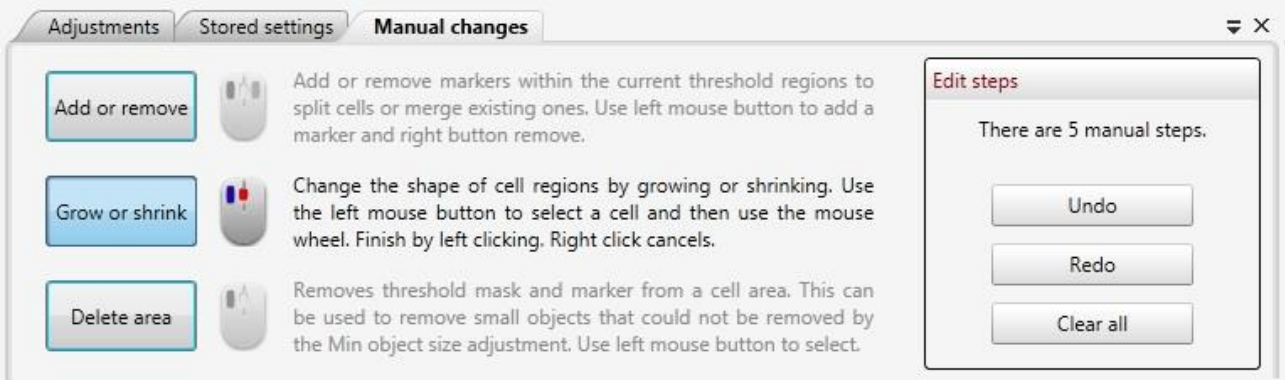
Adjustments
Stored settings
Manual changes
Add or remove
Add or remove markers within the current threshold regions to split cells or merge existing ones. Use left mouse button to add a marker and right button remove.
Grow or shrink
Change the shape of cell regions by growing or shrinking. Use the left mouse button to select a cell and then use the mouse wheel. Finish by left clicking. Right click cancels.
Delete area
Removes threshold mask and marker from a cell area. This can be used to remove small objects that could not be removed by the Min object size adjustment. Use left mouse button to select.
Edit steps
There are 5 manual steps.
Undo
Redo
Clear all

Figure 129: The Manual Changes window

11.3.2. Object Definition

Minimum Object Size (Figure 128) is used to manually adjust the size of the blue cell marker. The cell marker indicates the densest part of the cell.

11.3.3. Miscellaneous

Pre-smoothing (Figure 127) is a noise reduction function.

Checking Join Nearby Markers (Figure 127) results in two distinct cell cores being counted as one when they are very close to each other.

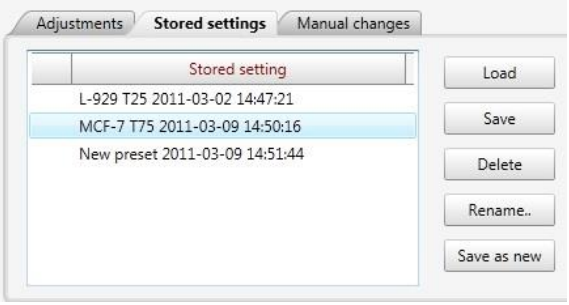

Figure 130: The Stored Settings window

11.4. Stored Settings

In the Stored Settings tab (Figure 130), threshold settings can be saved for future use. The settings will be dated and named New Presets when saved. The name can then be changed by highlighting the preset and clicking the Rename button. Stored settings can be applied to the current image with the Load button.

11.5. Manual Changes

In the Manual Changes tab (Figure 129), the threshold settings for single cells can be adjusted manually.

With the Add or Remove button identified cells can be split or merged.

With the Grow or Shrink button identified cells can be enlarged or shrunk.

With the Delete Area button identified cells can be deleted.

The editing performed with the Add or Remove button, Grow or Shrink button or Delete Area button can be undone, redone or all steps can be cleared using the editing buttons which are found to the right in the Manual Changes tab.

Several of these functions are found as a menu when clicking with the right mouse button in the image (Figures 131 and 132).

Figure 131: The adjustments menu that shows up when clicking outside identified cell areas

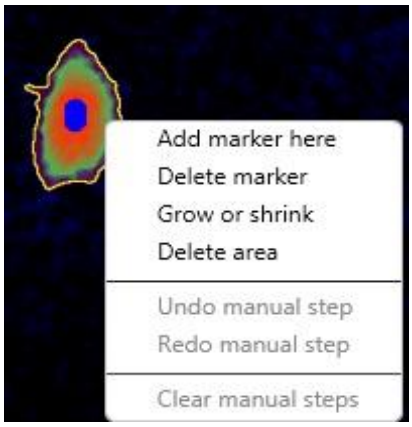

Figure 20: The adjustments menu that shows up when clicking on an identified cell area

11.6. Image Frame list

To the right of the Main viewing window, the images in the currently selected Project and Group are presented in the Image Frame list (Figure 133).

At the top of the window, first the Project and then the Group is presented. A different Project or Group can be selected with the drop menus.

The image that is highlighted will be shown in the Main Viewing Window.

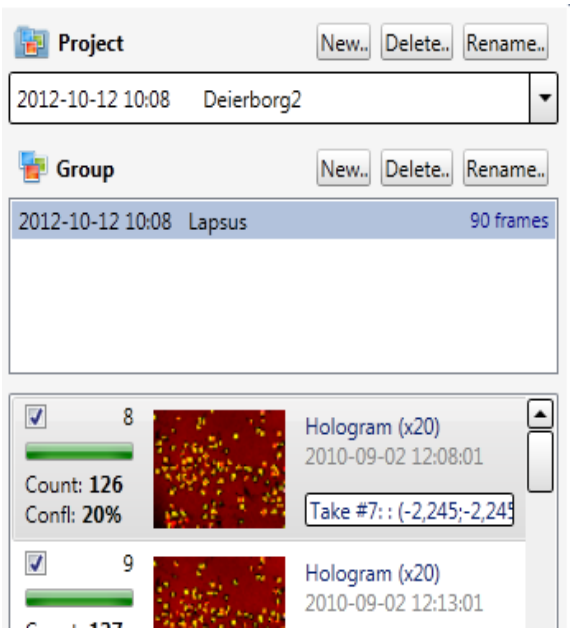

Figure 133: The Image Frame list

11.6.1. Image information

The frame number is given to the left of each image thumbnail.

A filling green bar to the left of the image thumbnail indicates that a process is ongoing. A filled green bar indicates that no process is ongoing.

The cells are automatically identified when the image is captured. The number of cells in the frame, as well as the confluency, are

given to the left of the image thumbnail.

To the right of each image thumbnail, the date and time of image capture are given. The take number and the well name of multiwell vessels as well as the stage objective coordinates are given to the right of each image thumbnail (Figure 134). In that same space the user can fill in information manually. The take number is the number of each time point in a timelapse sequence. If a timelapse is combined with a capture pattern, the images captured at the same timepoint will have the same take number.

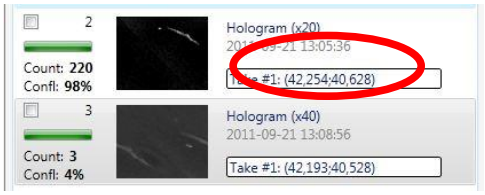

Figure 134: Take number information

11.6.2. Check and Delete

By clicking the right mouse button while hovering over an image thumbnail in the Image Frame list, a menu with the functions Check and Delete is accessed.

With the uppermost function Check, the boxes to the left of each image thumbnail can be checked (Figure 135). Either only the highlighted frames are checked or all frames. Alternatively the boxes can be checked manually by clicking the boxes with the left mouse button.
When Uncheck (Figure 135) is clicked, the boxes to the left of each image thumbnail are unchecked. Either only the highlighted frames are unchecked or all frames. Alternatively, the boxes can be unchecked manually by clicking the checked boxes with the left mouse button.

Figure 135: The Check menu

The Delete function (Figure 136) allows the user to delete one or more image frames. Either only the highlighted frames are deleted or all checked frames.

Figure 136: The Delete menu

11.6.3. Image Display

Below the Image Frame list there are further image display options (Figure 137). Checking or unchecking the boxes determines which images are displayed in the Image Frame list (Hologram and/or Phase Contrast and/or Only Checked).

When the box for Only Checked Frames is checked, only the checked frames will be displayed.

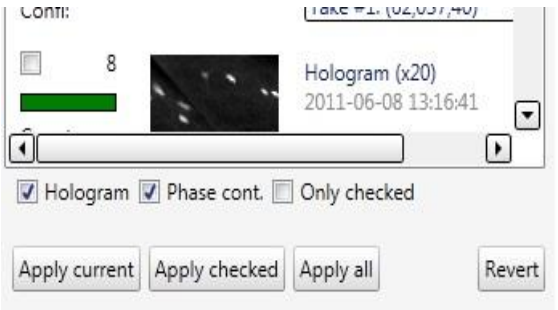

Figure 137: Image display options

11.6.4. Save changes

When the button Apply Current is clicked (Figure 137), the threshold settings of the image, currently shown in the Main Viewing

Window will be saved to the currently shown image.

When the button Apply Checked is clicked, the threshold settings of the image, currently shown in the Main Viewing Window, will be saved to the images which are checked in the The Image Frame list.

When the button Apply All is clicked, the threshold settings of the image, currently shown in the Main Viewing Window will be saved to all images in the Image Frame list.

When the button Revert is clicked, the latest change is undone.

Chapter 12. Cell Count

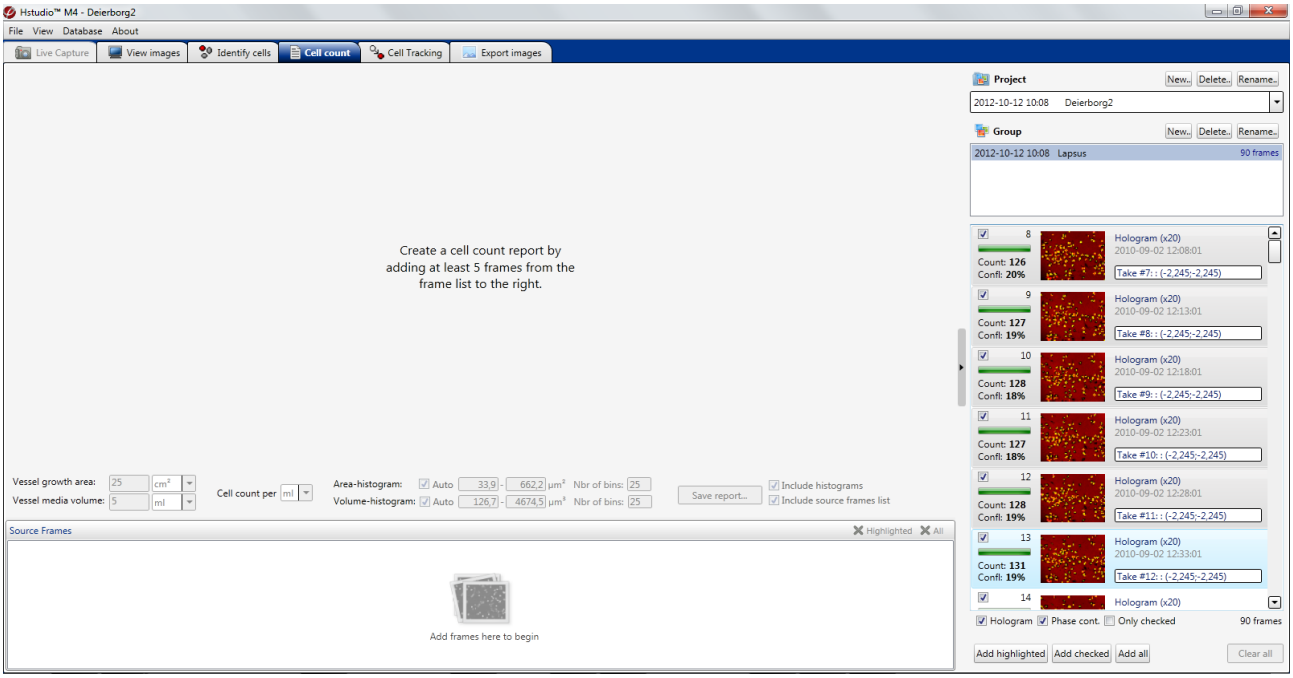

Figure 138: The Cell Count tab

In the Cell Count tab (Figure 138), data analysis is performed on holographic images, resulting in cell numbers, confluence and values for area and volume.

12.1. The Cell Count Report

12.1.1. Create a Cell count report

When first opening the Cell Count tab, the Main Viewing window presents information text (Figure 138), telling the user to add at least five image frames to create a cell count.

When five or more images have been added, a Cell Count Report is created and displayed in the Main Viewing window (Figure 139).

12.1.2. The contents of a cell count report

The report includes cell number and confluence as well as basic data concerning image capturing. Data on area and volume are presented as histograms in the report. The report contains data on:

- Number of cells in the vessel
- Number of cells per ml
- Confluence
- Report date
- Capture time points
- Vessel growth area
- Vessel media volume
- Total number of image frames used for the analysis
- The total area of the images
- The total number of cells in the images
- The number of cells that are placed on the image edge, and which are therefore not included in the morphological analysis, although they are included in the cell count.

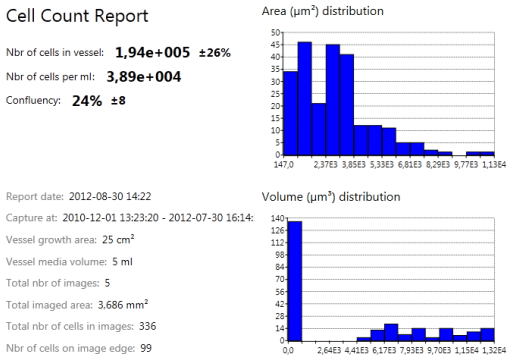
Cell Count Report
Nbr of cells in vessel: 1,94e+005 ±26%
Nbr of cells per ml: 3,89e+004
Confluency: 24% ±8
Area (µm²) distribution
Report date: 2012-08-30 14:22
Capture at: 2010-12-01 13:23:20 - 2012-07-30 16:14:
Vessel growth area: 25 cm²
Vessel media volume: 5 ml
Total nbr of images: 5
Total imaged area: 3,686 mm²
Total nbr of cells in images: 336
Nbr of cells on image edge: 99
Volume (µm³) distribution

Figure 139: Cell count report

12.2. Growth Area and Volume text boxes

Below the Cell Count Report, there are text boxes where the correct cell culture vessel growth area and the volume of the cell culture vessel medium content can be filled in (Figure 140).

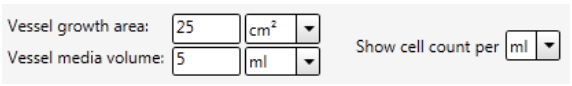

Figure 140: The text boxes for cell culture vessel area and volume

12.3. Save Report

Also below the cell count report there is a Save Report button (Figure 141).

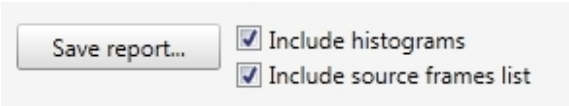

Figure 141: The Save Report button

12.4. The Source Frames list

Below the Cell Count Report there is a list of the frames which have been used for the cell count and analysis (Figure 142).

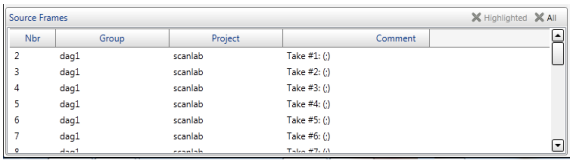

Figure 142: The Source Frames list

12.5. The Remove buttons

To the right in the Source Frames window there are Remove buttons (Figure 143). Clicking the buttons removes data from the cell count and the histograms.

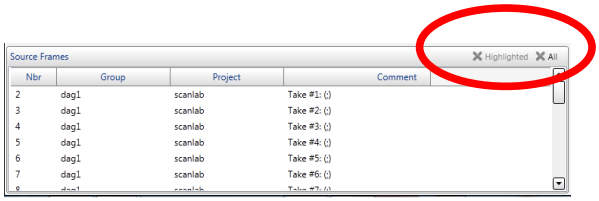

Figure 143: The Remove buttons

12.6. Image Frame list

To the right of the Main Viewing window, the images in the currently selected Project and Group are presented in the Image Frame list (Figure 144).

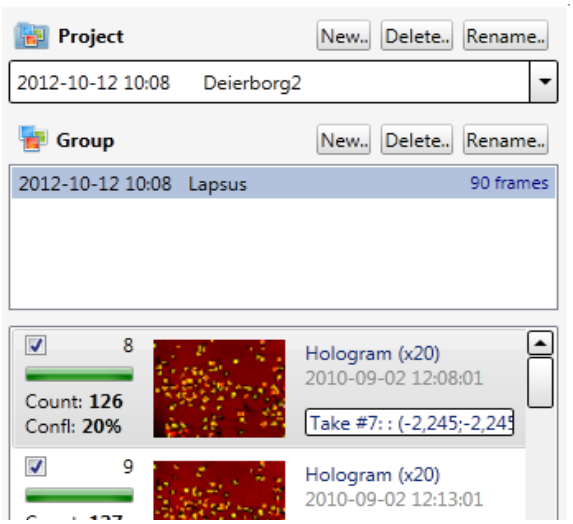

Figure 144: The Image Frame list

At the top of the window, first the Project and then the Group can be selected using the drop menus (Figure 138). The images belonging to the selected Group are shown in the Image Frame list. The image that is highlighted will be shown in the Main Viewing Window.

With the New buttons, new projects or groups can be created.

With the Delete buttons, Projects or Groups can be deleted.

With the Rename buttons, Projects or Groups can be renamed.

12.6.1. Image information

The image number is given to the left of each image thumbnail (Figure 144).
A filling green bar to the left of each image thumbnail indicates that a process is ongoing. A filled green bar indicates that no process is ongoing.

The number of cells in the frame as well as the confluency are given to the left of the image thumbnail.

To the right of each image thumbnail the date and time of image capture are given (Figure 145). The take number and the well name of multiwell vessels as well as the stage objective coordinates are given. In that same space the user can fill in information manually. The take number is the number of each time point in a timelapse sequence. If a timelapse is combined with a capture pattern, the images captured at the same timepoint will have the same take number.

Figure 145: Take number information

12.6.2. Check and Delete

By clicking the right mouse button while hovering over an image thumbnail in the Image Frame list, a menu with the functions Check and Delete is accessed (Figure 146).

146: The Check menu

Using Check, the boxes to the left of each image thumbnail can be checked. Either only the highlighted frames are checked or all frames. Alternatively the boxes can be checked manually by clicking the boxes with the left mouse button.

When Uncheck is clicked, the boxes to the left of each image thumbnail are unchecked. Either only the highlighted frames are unchecked or all frames. Alternatively, the boxes can be unchecked manually by clicking the checked boxes with the left mouse button.

The shift key can be used to highlight several consecutive images and the ctrl key to highlight non-consecutive images.

The Delete function (Figure 147) allows the user to delete one or more highlighted or deleted image frames.

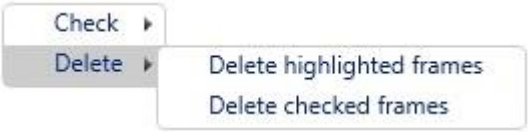

Figure 147 The Delete menu

12.6.3. Image display

Below the Image Frame list there are image display functions (Figure 148). Checking or unchecking the boxes determine which images are displayed in the Image Frame list (Hologram and/or Phase Contrast and/or Only Checked).

When the box for Only Checked Frames is checked, only the checked frames will be displayed in the list.

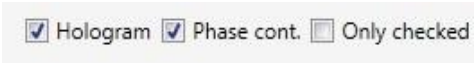

Figure 148: Image display functions

12.7. The Add buttons

Below the Image Frame list there are buttons to add image frame data to a plot (Figure 149) and beside the Source Frames list there are Remove buttons (Figure 143).

By clicking the Add Highlighted button (Figure 149), which is found below the Image Frame list, the data from one or more selected image frames can be added.

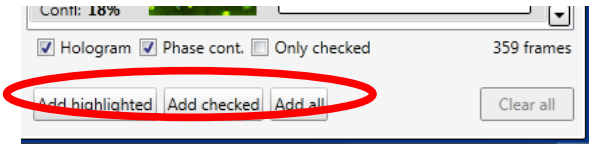

Figure 149: Buttons for adding data to plot or

Alternatively, the box to the left of each image can be checked and then the Add Checked button is clicked.
I
mages can be added to a maximum of totally 15 000 cells. The number of cells are given in the Cell Count Report (Figure 139).

12.8. The Clear All button

To the right of the Add buttons, there is a Clear All button (Figure 149), which removes all data from the Cell Count Report.

Chapter 13. Cell Tracking

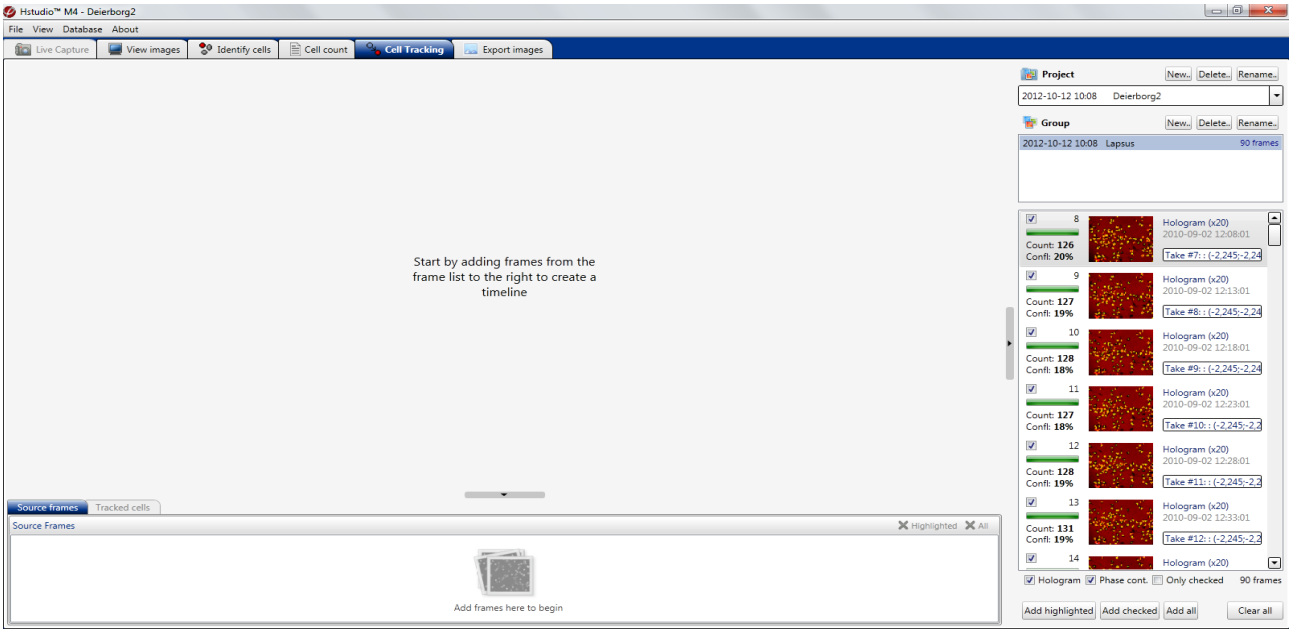

Figure 150: The Cell Tracking tab

For M4 Tracking only

Cells can be tracked through a timelapse sequence and analyzed both for movement and for morphology changes over time.

13.1. The Tracking tab

When the tab opens (Figure 150), a message (Figure 151) indicates that image frames from the frame list (Figure 152) must be added in order to create a timeline.

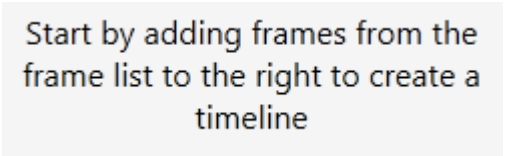

Figure 151: The Cell Tracking instruction text

13.1.1 Adding frames to the analysis

To the right of the Main Viewing window, the images in the currently selected Project and Group are presented in the Image Frame list (Figure 152). Highlight or check the images to be analyzed.

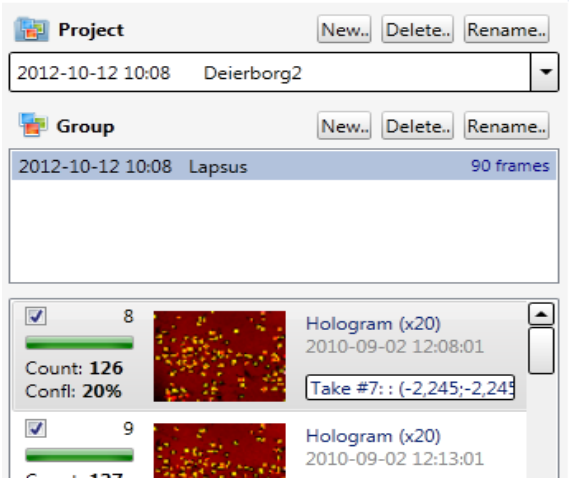

Figure 152: The Image Frame list

To the right of the Main Viewing window, the images in the currently selected Project and Group are presented in the Image Frame list (Figure 152).

At the top of the window, first the Project and then the Group can be selected using the drop menus (Figure 152). The images belonging to the selected Group are shown in the Image Frame list. The image that is highlighted will be shown in the Main Viewing Window.

Click the appropriate Add button (Figure 153). The Add buttons are found below the Image Frame List. The added image frames will be shown in the Source Frames list (Figure 154) which is found below the Cell Tracking window (Figure 155).

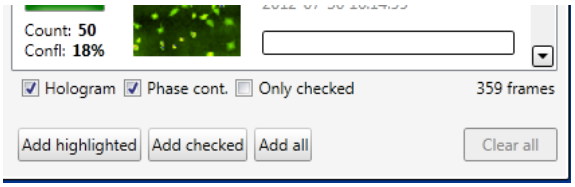

Figure 153: The Add buttons

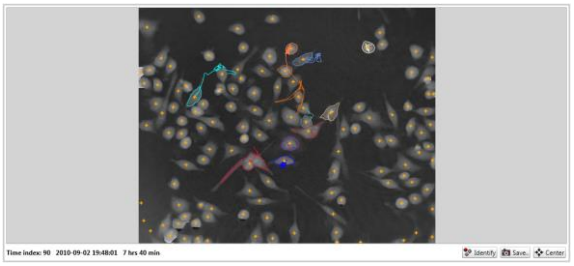

Figure 155: The Cell Tracking window

13.2. The Select Mode side window

13.2.1. Add cells

When the Add Cells function is activated in the Select Mode side window, cells can be added (Figure 156). Added cells are then

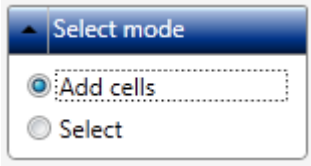

Figure 156: The Select Mode side window

followed from the frame where they were added until the end of the timelapse sequence.

To add a cell, click the center of an identified cell in the Cell Tracking window. The center of each identified cell is marked with a small orange **+** (Figure 157). If the identification is not satisfying, go to the Identify Cells tab, and adjust the cell identification.

The added cells will be listed in the Tracked cells list (Figure 154), which is found below the Cell Tracking window (Figure 155).

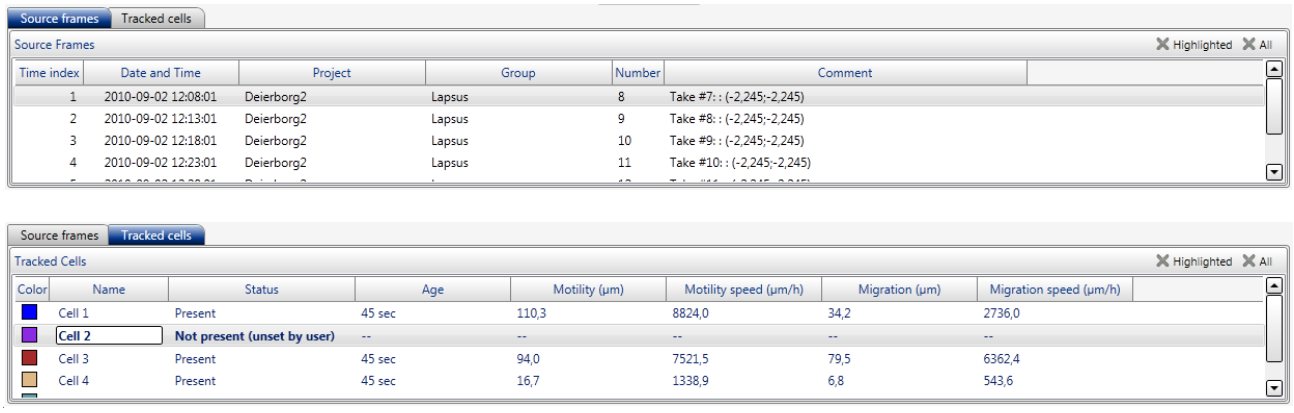

Figure 154: The Source Frames list and the Tracked Cells list

Figure 157: Clicking a cell to add

13.2.2. Select cells

When the Select Cells function is active in the Select Mode side window, cells can be selected in order to adjust their tracking. Activate Select in the Select Mode side window (Figure 156). Click on the cell to be adjusted. A new set of activities will then be available in the Change Tracking side window (Figure 158). The cell that will be adjusted is noted in the Change Tracking side window.

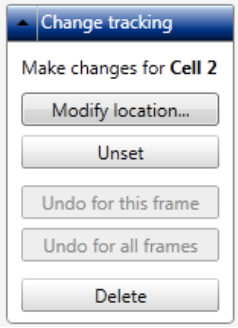

Figure 21: Change Tracking side window

13.3. The Change Tracking side window

13.3.1. Modify Location

Sometimes the software will track the wrong cell, e.g. when cells are moving very close to each other and then separate again. This needs to be adjusted manually.

The Modify Location button allows the user to switch the tracking from one cell to another. After selecting the cell to be changed, click Modify Location button in the Change Tracking side window (Figure 158). Then click the cell that should actually be followed instead of the selected cell. The colored border will be transferred from the previously tracked cell to the currently tracked cell (Figure 159). The software will recalculate the tracking automatically from the present image frame and forward through the time lapse.

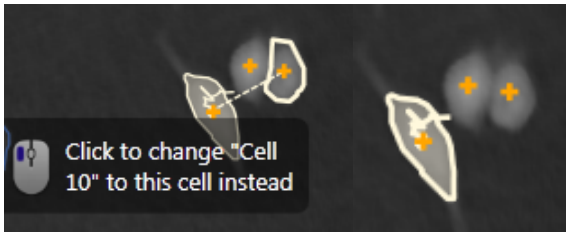

Figure 159: Transfer the tracking from one cell to another. The left image shows the selected cells, and the right image shows how the tracking is transferred.

13.3.2. Unset

Sometimes a tracking needs to be discontinued, e.g. when a cell leaves the image area.

By clicking the Unset button in the Change Tracking side window (Figure 158), the tracking of a selected cell will be discontinued from the present frame.
A new button, Set location, will appear in the Change Tracking window (Figure 160).

13.3.3. Set location

When a cell has been unset it will still be present in the Tracked Cells list, but noted as not present. In order to resume the tracking in a different frame, select the cell in the Tracked Cells list and then click the Set location button (Figure 160). Thereafter click the cell where the tracking should be resumed.

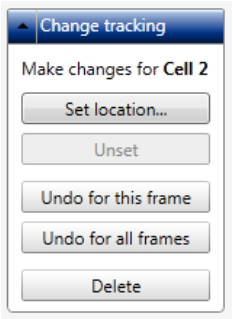

Figure 160: Set Location

13.3.4. Undo for this frame

When the user has made a change to the cell tracking, that change can be undone for the current frame by clicking Undo For This Frame (Figure 161). The manual change in the current frame will be undone, and the tracking will be recalculated according to the change in settings.

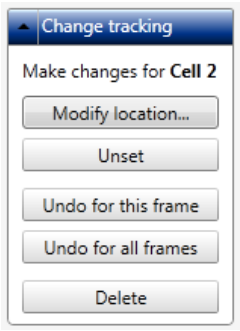

Figure 161 Undo for this frame

13.3.5. Undo for all frames

Manual changes to the cell tracking for the currently selected cell can be undone in all frames by clicking Undo For All Frames (Figure 161). The manual changes in all frames will be removed and the tracking will be recalculated according to the change in settings. Only the very first definition will be kept.

13.3.6. Delete

Clicking the Delete button (Figure 161) will delete the currently selected cell from the tracking. It can be added again by activating the Add Cells function in the Select Mode side window (Figure 154).

13.4. Track

When the Track button is clicked, the tracking will be recalculated (Figure 162).

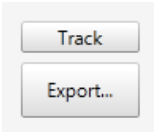

Figure 22: Track and Export buttons found to the right of the Cell Tracking window

13.5. Export

The raw tracking data can be exported into an xml file by clicking the Export button (Figure 162).

13.6. Identify

The Identify button is a quick way to enter the Identify cells tab if the image needs to be adjusted. The button is found below the Cell Tracking window (Figure 163)

Figure 23: Identify, Save and Center buttons found below the Cell Tracking window

13.7. Save

The image currently displayed in the cell tracking window can be exported by clicking the Save button found below the Cell Tracking window (Figure 163).

13.8. Center

The Center button moves the image in the Cell Tracking Window to the center of the display area. The button is found below the Cell Tracking window (Figure 163).

13.9. Timeline

There is a Timeline below the Cell Tracking window (Figure 165). By moving the small gray bar, the cell movements will be displayed in the Cell Tracking window.

As the cells are followed through the frames, tracks showing their movements will be displayed (Figure 166).

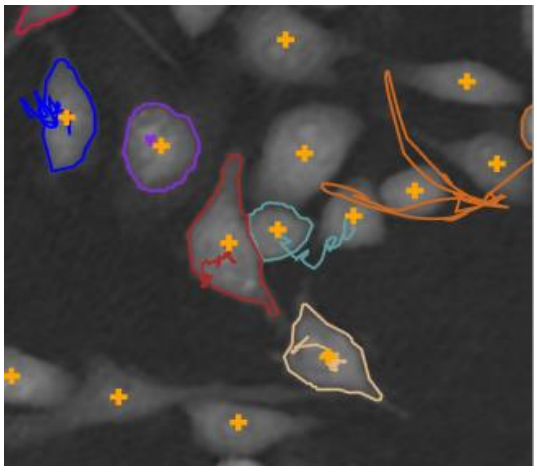

Figure 166: Tracks showing cell movements

Figure 165: Timeline for cell tracking

13.10. Plot Movement Tab

The Plot Movement tab will not be active until at least one cell has been added to the tracking.

13.10.1. Spatial Tracking diagram

When cells have been traced through a timelapse sequence, the direction of the movement can be seen in a diagram (Figure 166). Both the average movement for all cells and the movements of each individual cell can be shown (Figure 167).

13.10.1. Spatial Tracking diagram

When cells have been traced through a timelapse sequence, the direction of the movement can be seen in a diagram (Figure 167). Both the average movement for all cells and the movements of each individual cell can be shown (Figure 168).

The display of the Spatial Tracking diagram can be adjusted manually by using the functions found below the diagram window (Figure 169). When Auto Scale is unchecked, it is possible to set the minimum and maximum axis values manually. By checking symmetric or square it is possible to change the shape of the diagram.

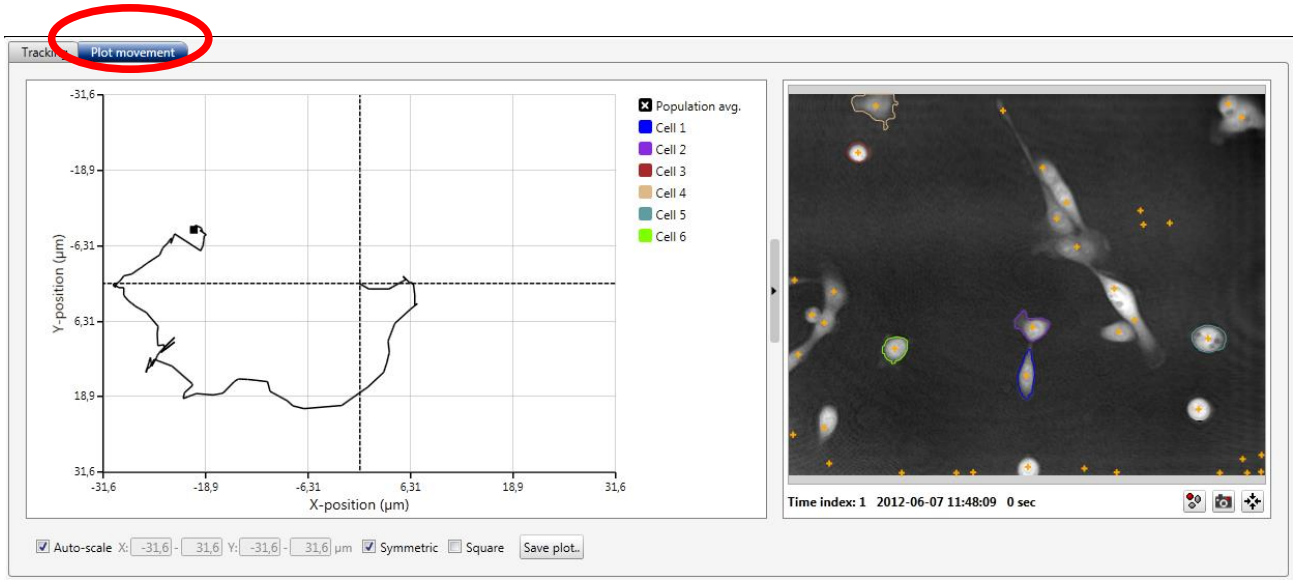

Figure 24: The Plot Movement tab

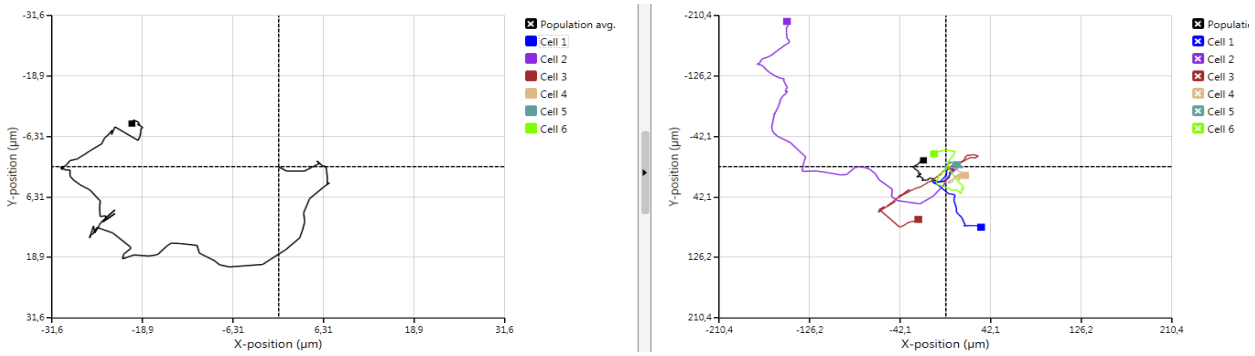

Figure 25: Diagrams showing both the average cell movement (right image) and the movement of individual cells (left image)

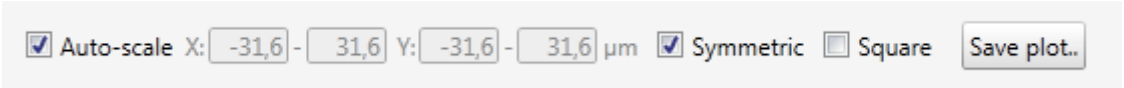

Figure 26: Adjustment functions for the Spatial Tracking diagram

13.11. Source frames tab

Below the Tracking window there are two tabs with lists (Figure 170). The Source frames list contains information on the frames that are included in the tracking analysis.

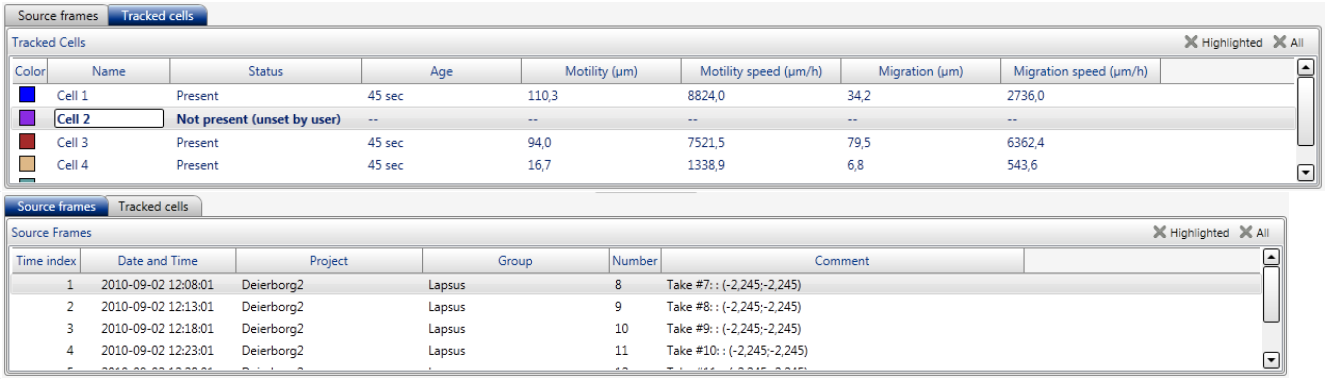

Figure 170: The Source Frames list and the Tracked Cells list

13.11.1. Delete frames

By highlighting one or several frames in the list, it is possible to remove them from the analysis by clicking **X** Highlighted (Figure 170). When **X** All is clicked, all frames will be removed from the list.

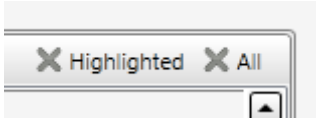

Figure 171: Functions to remove frames from the Source Frames list

13.12. Tracked cells tab

Below the Tracking window there are two tabs with lists (Figure 170). The Tracked cells list contains information on the individual cells that are followed through a timelapse set of captures. Information such as Motility and Migration is given in a table. It is also noted if the cell is present in the current frame.

13.12.1. Naming individual cells

In the Name-column of the Tracked cells list, the cells are named with numbers in the order in which they have been added. By clicking the cell number, a textbox is activated where any cell name can be entered (Figure 172).

13.12.2. Deleting cells

By highlighting one or several cells in the list, it is possible to remove them from the analysis by clicking **X** Highlighted (Figure 171). When **X** All is clicked, all frames will be removed from the list.

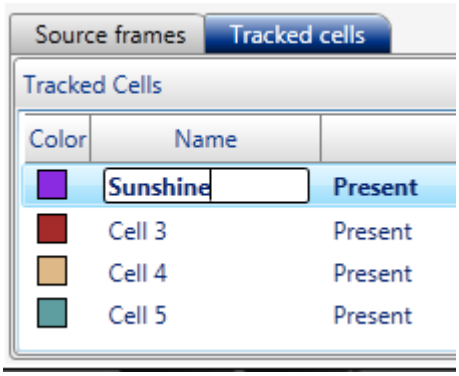

Figure 172: Renaming a cell in the tracked cells list

Chapter 14. Export Images

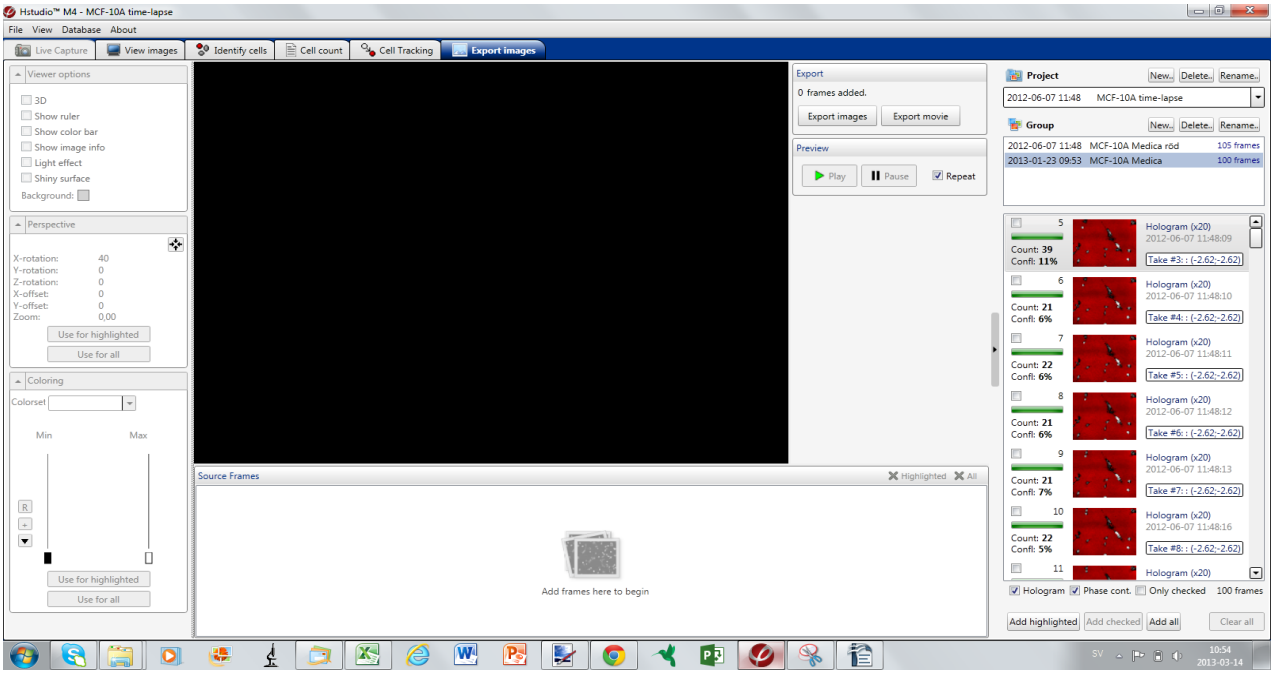

Figure 27: The Export Images tab

In the Export Images tab (Figure 173), image frames can be edited and exported either as individual images in several standard formats or as AVI movies.

The side windows become active when one or several images are added.

14.1. Viewer Options

The Viewer Options side window (Figure 174), which is found to the left of the Main Viewing window, is used to change how the image is displayed. Some of the functions are inactive when a phase contrast image is viewed.

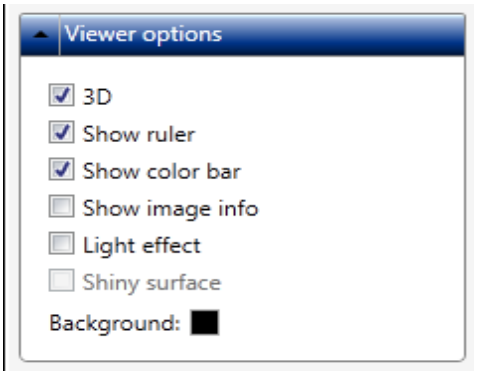

Figure 28: Viewer Options side window

Checking 3-D displays the image as a 3-dimensional representation.

Checking Show Ruler displays a horizontal scale bar representative of the distance in X and Y.

Checking Show Color Bar displays a vertical scale bar representative of the height in Z.

Checking Show Image Info displays additional information associated with the image such as (Figure 110):

Project: specifying in which project the image is located

Group: specifying in which group the image is located.

Nbr: Specifying which number the image has in the group.

Type: Specifying if the image is holographic or phase contrast.

Date: specifying the capture date and time of the image

Checking Light Effect applies an artificial light source to the image which may sometimes render an improved image.

Checking Shiny surface applies a change in the surface image display that sometimes renders a better image. Shiny surface is only active when Light Effect is checked.

Background can be used to change the background color in the Main Viewing window.

14.2. Perspective

The Perspective window (Figure 175) shows how the image has been moved, flipped and zoomed. These changes can also be applied to the added images that are highlighted or to all added images.

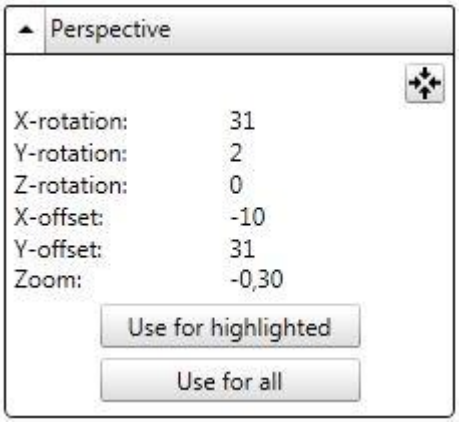

Figure 175: The Perspective side window

To zoom the image, left click the image in the Main Viewing window and then use the mouse scroll button.

To move the image to a desired location in the Main Viewing window, click, hold and drag the image using the left mouse button.

To flip and move the 3-D image, click, hold and drag the image using the right mouse button.

14.3. Coloring

The Coloring side window (Figure 176) is used to adjust the holographic image color display in the Main Viewing window and to adjust the image to optimally display the image dynamics.

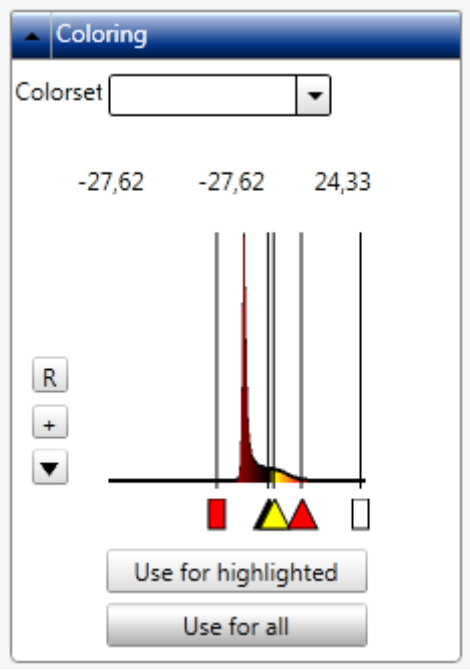

Figure 176: The Coloring side window

A set of colors that are saved together is called a colorset. A previously saved colorset can be used with the current image by making a selection in the Colorset list which is found at the top of the Coloring side window (Figure 176).

By left clicking the R-button (Figure 176), the coloring in the image is rescaled to better utilize the optimal dynamic range of the image. This button needs to be operated every time the image coloring is off.

To add a new color to the colorset, left click the plus button (Figure 176) and select a new color. A colored triangle representing the new color will appear beneath the histogram (Figure 175). Alternatively, right click the x-axis at the position where a new color is wanted and select Add Color from the menu that appears.

Change the color by using the right mouse button to click on a colored triangle beneath the histogram and select a new color, alternatively left click the arrow button and select Change Color (Figures 176 and 177). To change the color span, left click and move the desired colored triangle beneath the histogram using the cursor (Figure 176).

To save a new colorset with the current color settings, left click the arrow button and choose Save As (Figure 176).

To save the current color settings to a previously saved colorset, left click the arrow button and choose Save (Figure 176). Note that this will overwrite the settings previously saved to this colorset.

To delete a colorset, select it in the Colorset list by left clicking it. Then left click the arrow button and select Delete Colorset (Figure 176).

Coloring settings can be applied to the added images that are highlighted or to all added images.

To move the image to a desired location in the Main Viewing window, click, hold and drag the image using the left mouse button.

To flip and move the 3-D image, click, hold and drag the image using the right mouse button.

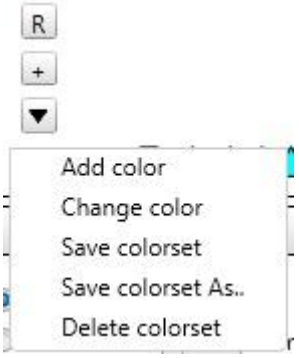

Figure 29: Additional functions found in the Coloring side window

14.4. Main Viewing window

The Main Viewing window contains a view of the currently active added image as well as thumb nails of all the added images (Figure 178). By clicking a thumb nail, a new image will be displayed in the Main Viewing window.

The thumb nails display information about frame number, group affiliation and date and time of capture.

In order to change the image sequence, the thumb nails can be repositioned by clicking and dragging,

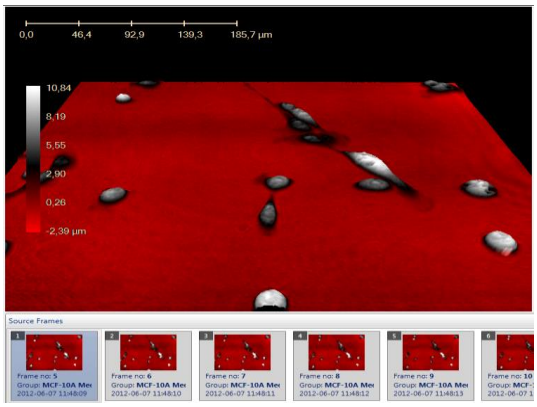

Figure 30: The Main Viewing window

14.5. Export

In the Export window (Figure 179), the added images can be exported either individually as images or together as an AVI movie.

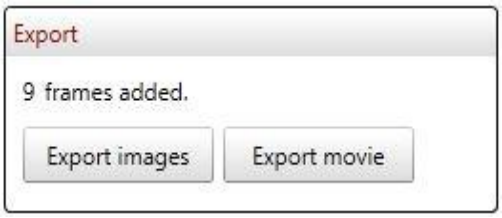

Figure 31: The Export window

14.5.1. Export Images

Clicking the Export Images button will open an export window (Figure 180). The destination folder can be selected by browsing. When the box is checked, frame comments will be added to the file name. The Image format can be selected in the drop menu. There are five different formats to choose from: bitmap, GIF, jpg, png and TIFF.

When the box for Export Raw Images is checked, no coloring or 3-D will be displayed in the exported image.

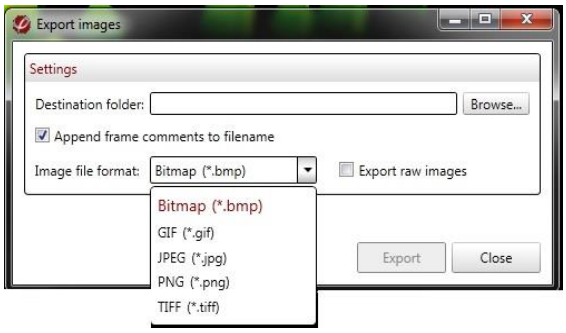

Figure 32: The Export images window

14.5.2. Export Movie

Clicking the Export Movie button will open an export window (Figure 181). The destination folder can be selected by browsing.

By moving the slide bar it is possible to set the number of frames per second that will be shown in the AVI movie.

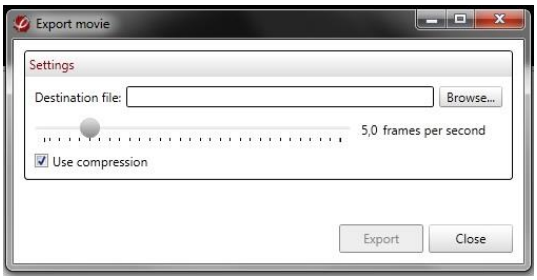

Figure 33: The Export Movie window

14.6. Preview

When the preview button Play (Figure 182) is clicked, a preview of the movie is shown. The number of slides per second is not shown as in the finished movie, but is rather at a set speed.

Figure 34: The Preview window

14.7. Remove

By highlighting one or several thumb nails in the list, it is possible to remove frames from the movie by clicking **X** Highlighted (Figure 183). When **X** All is clicked, all frames will be removed from the list. The X functions are found in the upper right corner of the thumb nails window.

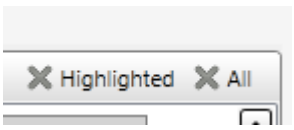

Figure 35: The Remove functions

To the right of the Main Viewing window, the images in the currently selected Project and Group are presented in the Image Frame list (Figure 184).

At the top of the window, first the Project and then the Group can be selected using the drop menus (Figure 184). The images belonging to the selected Group are shown in the Image Frame list. The image that is highlighted will be shown in the Main Viewing Window.

With the New buttons new projects or groups can be created.

With the Delete buttons Projects or Groups can be deleted.

With the Rename button Projects or Groups can be renamed.

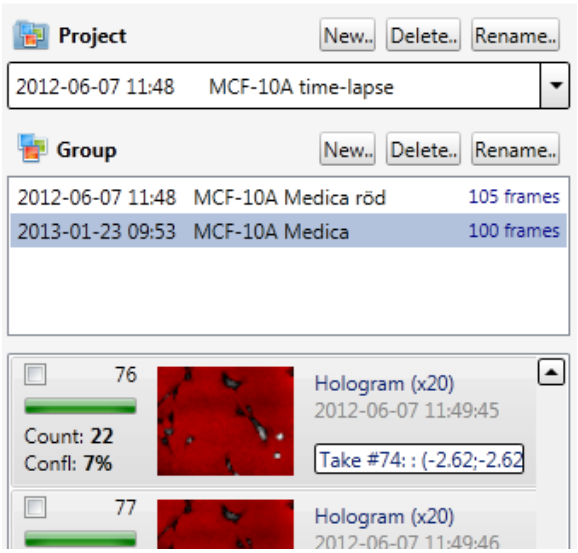

Figure 36: The Image Frame list

14.8.1. Image information

The image number is given to the left of each image thumbnail (Figure 185).
A filling green bar to the left of each image thumbnail indicates that a process is ongoing. A filled green bar indicates that no process is ongoing.

The number of cells in the frame as well as the confluency are given to the left of the image thumbnail.

To the right of each image thumbnail, the date and time of image capture are given (Figure 185). The take number and the well name of multiwell vessels, as well as the stage objective coordinates are given in a text box. In that same space the user can fill in information manually. The take number is the number of each time point in a timelapse sequence.

If a timelapse is combined with a capture pattern, the images captured at the same timepoint will have the same take number.

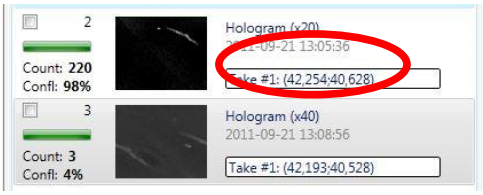

Figure 185: Take number information

14.8.2. Check and Delete

By clicking the right mouse button while hovering over an image thumbnail in the Image Frame list, a menu with the functions Check and Delete is accessed (Figure 186).

186: The Check menu

Using Check, the boxes to the left of each image thumbnail can be checked. Either only the highlighted frames are checked or all frames. Alternatively the boxes can be checked manually by clicking the boxes with the left mouse button.

When Uncheck is clicked, the boxes to the left of each image thumbnail are unchecked. Either only the highlighted frames are unchecked or all frames. Alternatively, the boxes can be unchecked manually by clicking the checked boxes with the left mouse button.

The shift key can be used to highlight several consecutive images and the ctrl key to highlight non-consecutive images.

The Delete function (Figure 186) allows the user to delete one or more highlighted or deleted image frames.

Figure 186: The Delete menu

14.8.3. Image display

Below the Image Frame list there are image display functions (Figure 187). Checking or unchecking the boxes determines which images are displayed in the Image Frame list (Hologram and/or Phase Contrast and/or Only Checked).

When the box for Only Checked Frames is checked, only the checked frames will be displayed in the list.

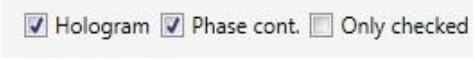

Figure 187: Image display functions

14.9. Add frames

Below the Image Frame list there are buttons to add image frames to the Main Window (Figure 188).

By clicking the Add Highlighted button (Figure 188) which is found below the Image Frame list, the data from a single image frame can be added when it is highlighted in the Image Frame list.

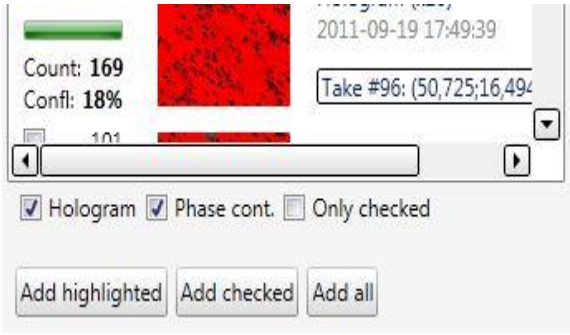

Figure 188: Buttons for adding images to the Main Window

The data from several image frames can be added simultaneously by highlighting them and clicking the Add Highlighted button. Alternatively, the box to the left of each image can be checked and then the Add Checked button is clicked.

All image frames can be added by clicking the Add All button.

Chapter 15. Main top menu

There are four sub-menus in the Main Top Menu: File, View, Database and About (Figure 189).

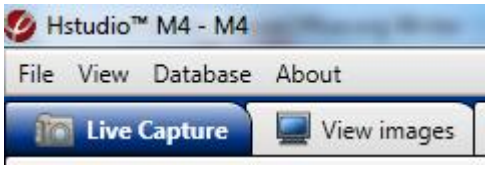

Figure 189: The Main Top menu

15.1. File

Under Files you can handle image projects or exit the program.

15.1.1. Projects

The Projects menu (Figure 190) can be used to create a new project, delete projects or rename projects. The functions are applied to the project that is currently selected in the Image Frame list to the right of the Main Viewing window.

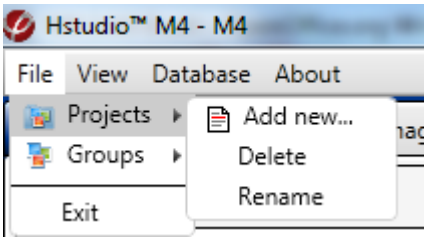

Figure 190: The Projects menu

15.1.2. Groups

The Groups menu (Figure 191) is used to create a new group within the currently selected project, and to delete or rename groups that are currently selected in the Image Frame list to the right of the Main Viewing window.

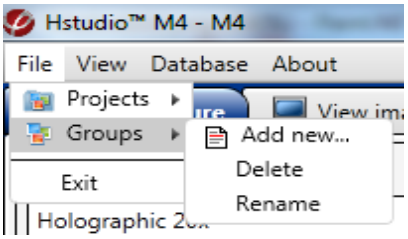

Figure 191: The Groups menu

15.1.3. Exit the program

When the Exit function (Figure 192) is clicked, the program closes down.

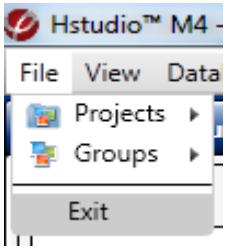

Figure 37: The Exit function

15.2. View

View contains an Auto Focus monitor function (Figure 193) that can be used to display the actual focus status (Figure 194). A sharp V-shape of the curve indicates a well-functioning auto focus which will result in focused images.

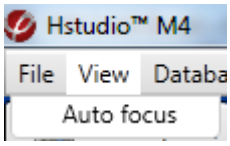

Figure 193: The Auto Focus function

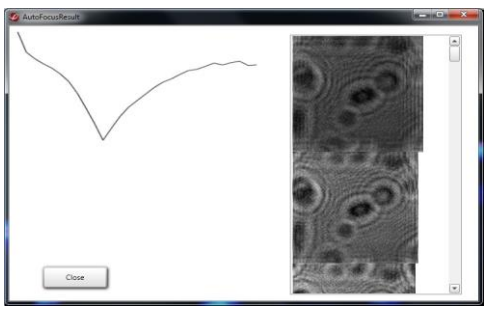

Figure 38: The focus status window

15.3. Database

In the Database menu (Figure 195), data from the image database can be exported or imported.

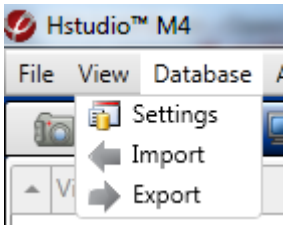

Figure 195: The Database menu

15.3.1. Database settings

When Database Settings is activated, a window opens where the database pathways are shown (Figure 196).

The Change Location button (Figure 196) will move the data base from the current hard drive. The HoloStudio data base is routinely placed on the c-drive of the computer, but sometimes it is better to store the database on a different hard drive.

The Clear button (Figure 196) will clear all data from the data base.

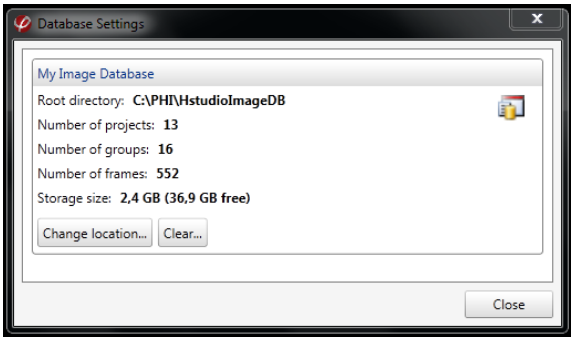

Figure 196: The Database Settings window

15.3.2. Database import

When Database Import (Figure 195) is selected, a Database Import window opens (Figure 197). By using the browse function, a folder which contains database data can be selected. When a folder has been selected, the projects and groups in the folder will be made available for selection in the Database Import window (Figure 198). The projects, groups or images are selected by ticking the box belonging to each item. A message will appear when the import is complete.

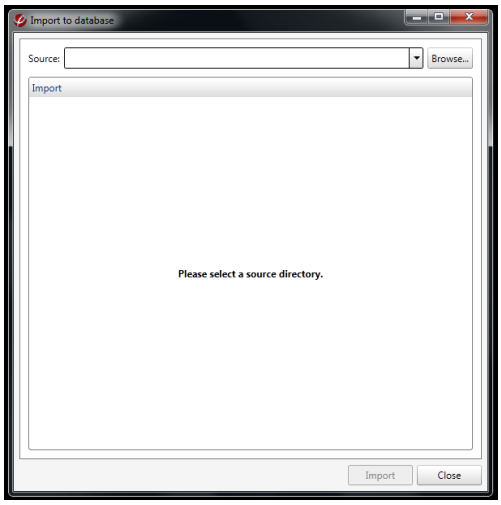

Figure 197: The Database Import window

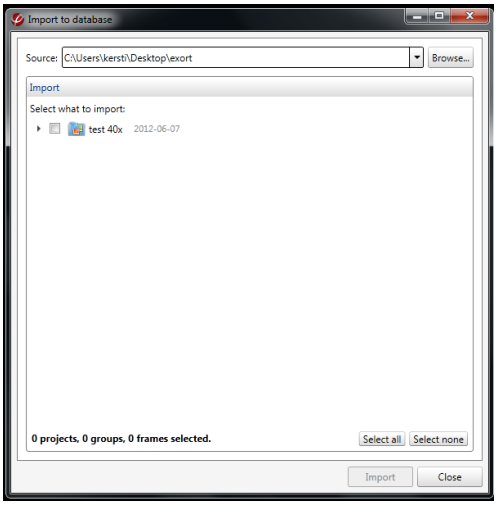

Figure 198: The Database Import window after browsing for a folder containing database data

15.3.3. Database export

When Database Export (Figure 195) is selected, a Database Export window opens (Figure 199). By using the browse function, a folder which will contain the exported database data can be created. When a folder has been selected, the projects and groups in the folder will be made available for selection in the Database Export window (Figure 200).

The projects, groups or images are selected by checking the box belonging to each item. This process is used to make backups of the data base. A message will appear when the export is complete.

Figure 199: The Database Export window

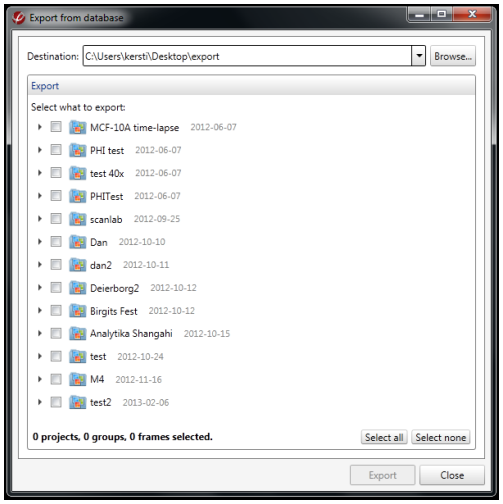

Figure 200: The Database Export window

15.4. About

Clicking the About function opens a window with information about the software version and copyright (Figure 201).

Figure 201: The About window

Manual PART FOUR

Chapter 16. Troubleshooting

16.1. Focusing

16.1.1. The Live Capture tab is inactive

Why? The software cannot find the HoloMonitor.
Fix: Connect the HoloMonitor, switch it on and restart HoloStudio.

16.1.2. It is impossible to focus the live image

Why? The cell culture vial is scratched or smudged.
Fix: Even wiping it clean will probably not help much as traces of dirt will still persist. Instead, avoid touching the top and bottom surfaces of the cell culture vessel when handling it, even when wearing gloves. Avoid anything that might scratch the surfaces. Cell culture plastic is very easily scratched.

Why? There is condensation on the inside of the cell culture vessel.
Fix 1: If the cell culture vessel is a flask, tighten the cap and the gently rinse the condensation away with the cell culture medium. Avoid getting medium into the cap. If the cell culture vessel is a multiwell plate or a petri dish, allow the vessel to reach room temperature, alternatively switch lids to a new lid or try working without lid. NOTE! Working without the lid is a non-sterile procedure!
Fix 2: If the cell culture medium is replaced with cold PBS immediately after taking the multi well plates and petri dishes from the incubator, there will not be condensation. Replace the PBS with medium again after the analysis.

Why? The laser intensity is not optimal for the chosen cell sample.
Fix: Calibrate the laser to optimize it for the current sample. Under the Live Capture tab, open the Calibration side window, and press the Calibrate laser button. The cell sample should be placed on the stage while doing this.
Why? If the cells are very thin or very sparsely distributed in the vessel, the auto focus has difficulties to find focus.
Fix: Set the software focus manually instead of automatically. Activate the manual focus mode by clicking the Manual button in the Software focus side window in the Live Capture tab. Thereafter drag the slider to the green area in the focus bar, and then set the hardware focus with the focusing wheel. As thin cells have a very narrow focal distance focusing has to be performed with great care.

Why? The instrument was not switched on for 30 minutes before use at room temperature or put in an incubator at least three hours before use. If the instrument is warming up, the focus will change all the time and the focus settings will need to be adjusted continuously.
Fix: Allow the instrument the warming up period.

16.1.3. The cells are very bright and blurry showing no inner structures

Why: The image dynamics are not optimally set.
Fix: Press the R-button by the histogram in the Coloring side window.

16.1.4. The cell image is completely white

Why: The image dynamics are not optimally set.
Fix: Press the R-button by the histogram in the Coloring side window.

16.1.5. The cell image is black

Why? The images are either zoomed in or out too much.
Fix: Use the mouse scroll to scroll back.

Why? The image dynamics are not optimally set.
Fix: Press the R-button by the histogram in the Histogram side window.

16.1.6. The live image focus was OK, but it slowly turned bad and now it cannot be set again

Why? There is probably a drop of water hanging from the top/lid of the cell culture vessel. As the drop grows larger it will increasingly disturb the image focus.
Fix: Gently tilt the cell culture vessel and make the drop slide to the side of the vessel.

Why? The instrument was not switched on for 30 minutes before use at room temperature or put in an incubator at least three hours before use. If the instrument is warming up, the focus will change all the time and the focus settings will need to be adjusted continuously.
Fix: Allow the instrument the warming up period.

16.2. Capture

16.2.1. The cell image in the Main Viewing window is white

Why? The image dynamics is not optimal.
Fix: Click the R-button in the Histogram side window.

16.2.2. It is impossible to capture an image as the capture button is inactive

Why? The capture button is inactive when no project and group has been selected. The program then does not know where to store the captured images.
Fix: Select a Project and a Group.

16.2.3. In a series of captured images not all images were good

Why? There might have been a scratch or a smudge on the vessel where the bad images were captured.
Fix: Capture extra images and then discard the bad ones.

Why? Floating cells disturbed the automatic software focus.
Fix: Follow the instructions in section 4.5. to recalculate the software focus.

Why? The time between frames (i.e. the Settle Time After Each Stage Movement) was too short. It is not possible for the software to focus while the cell culture medium is still moving.
Fix: Set a higher value for Settle Time After Each Stage Movement.

Why? The vessel bottom is not 100% horizontal and this creates a variation in physical distance from the objective.
Fix: Make sure that the vessel is correctly placed on the stage/adaptor and adjust the hardware focus with the focus wheel again.

16.3. View Images

16.3.1. No image frames are visible in the Image Frame list

Why? No Project and Group has been selected.
Fix: Select a Project and a Group.

Why? If the Only Checked box is checked, only checked images will be displayed. If no image frames in the current Group has been checked, no frames will be visible.
Fix: Uncheck the Only Checked box.

16.3.2. The cell image in the Main Viewing window is white

Why? The image dynamics is not optimal.
Fix: Click the R-button in the Histogram side window.

16.4. Cell Identification

16.4.1. No image frames are visible in the Image Frame list

Why? No Project and Group has been selected.
Fix: Select a Project and a Group.

Why? If the Only Checked box is checked, only checked images will be displayed. If no image frames in the current Group has been checked, no frames will be visible.
Fix: Uncheck the Only Checked box.

16.4.2. The cell image in the Main Viewing window is white

Why? The image dynamics is not optimal.
Fix: Click the R-button in the Histogram side window.

16.4.3 The automatic cell identification looks strange

Why? The chosen threshold setting method might not suit the cells.
Fix: Try a different threshold setting method.

Why? If the cells grow at a very high density, the threshold setting methods are not able to identify individual cells. If cells are growing very close they cannot be separated out.
Fix: Adjust the threshold setting using the Adjustment slide bar (see section 10.4). Make sure that only the background and not any cells are defined as background. Then use the confluency or the cell dry mass parameter to determine the amount of cells instead of the cell number.

Why? There is background noise in the image that causes the background to be set at an incorrect level.
Fix 1: Make sure that background and laser are calibrated prior to analysis.
Fix 2: Capture extra images and then discard the bad ones.

16.4.4. Some cells are incorrectly segmented as two or more

Why? The minimum object size is set too low.
Fix: Adjust the Min object size using the slider in the Object definition box under the Adjustment tab so that each cell has one blue marker.

16.4.5. Two cells are segmented as one

Why? The minimum object size is set too high.
Fix: Adjust the Min object size using the slider in the Object definition box under the Adjustment tab so that each cell has one blue marker.

Why? The cell confluency is very high and the cells are difficult to separate out.
Fix: Separate the two cells by using the Add or remove button under the Manual changes tab.

16.5. Cell Count

16.5.1. No image frames are visible in the Image Frame list

Why? No Project and Group has been selected.
Fix: Select a Project and a Group.

Why? If the Only Checked box is checked, only checked images will be displayed. If no image frames in the current Group has been checked, no frames will be visible.
Fix: Uncheck the Only Checked box.

16.6. Export images and movies

16.6.1. No image frames are visible in the Image Frame list

Why? No Project and Group has been selected.
Fix: Select a Project and a Group.

Why? If the Only Checked box is checked, only checked images will be displayed. If no image frames in the current Group has been checked, no frames will be visible.
Fix: Uncheck the Only Checked box.

16.6.2. The cell image in the Main Viewing window is white.

Why? The image dynamics is not optimal.
Fix: Click the R-button in the Histogram side window.

Index

3-D 25, 41, 55, 79
3-D 19
3-D 47
a user guide 17
Adaptive gaussian 30, 63
Adaptive mean 30, 63
Add All 85
Add All 40
Add button 35, 37, 73
Add Cells 37, 73
Add Checked 40, 71
Add Highlighted 71, 85
Add Highlighted 40
Add or Remove 31
Add or Remove button 64
Adjustment slide bar 63
Adjustment, slide bar 30
Adjustments 62
Adjustments window 29, 30
Amplitude 19, 25, 47
Apply All 32, 67
Apply Checked 32, 67
Apply Current 32, 66
apture a timelapse 52
area 68
Auto Focus 86
Auto Scroll 60
Auto-apply Changes 33, 61
Auto-apply Changes 32
Automatic 50
Automatic software focusing 49
back ups 88
Background 48
Background 80
Background calibration 51
Calibrate Objective 50
Calibrate the laser 89
Calibration Settings 58
Calibration side window 51
Camera Properties side window 51
camera, hologram 50
Capture 51, 52
Capture button 22
Capture window 22
cell core, adjust the size of the 30
Cell Count 10, 44
Cell Count Report 35, 68
Cell Count tab 34
cell culture vessel growth area 35

cell identification....29
cell identification settings....32
cell marker....64
cell number....29, 68
Cell ref index....51, 58
Cell Tracking....10
Cell Tracking....37
Cell Tracking,....44
Center button....24, 53
Center image....19
change color....49, 81
Change color....26
Change Color....56
Change Location....87
change the color span....49, 56, 81
Change Tracking....38, 74
Check....59, 66, 70, 84
clear all....31
Clear All button....71
Clear button....87
Close down....17
color settings, save....27
color settings, save the current....21
color span, change....26
color span, change the....21
color, add new....26
color, Change the....21
color, new....20
Coloring....42, 48, 55, 80
coloring, rescaled....26
colorset....20, 26, 42, 48, 55, 80
Colorset list....26, 42, 48, 55, 80
colorset, delete....27
colorset, delete a....21
colorset, save a new....21
colorset, save to image....27
colorset, saved....26
condensation....89
confluence....29, 68
confluency....59, 65
Count cells....34
culture vessel growth area....69
Database Export....88
Database Import....87
Database menu....87
Database Settings....87
Date....25, 42, 55, 80
date and time....66
date and time of image capture....59
Delete....66, 70, 75, 83, 84

Delete 60
delete a colorset 49, 56, 81
Delete Area 31
Delete Area button 64
Double adaptive gaussian 30, 63
Double adaptive mean 30, 63
Double otsu 30, 63
Exit 86
Export 75
Export button 39
Export Images 10, 43, 44, 82
Export Movie 82
Export Movie 43
Export Raw Images 43, 82
Exposure Time 50
FFT 19, 47
Files 86
flip and move a holographic 3-D image 24, 27
flip and move the 3-D image 41, 80, 81
flip and move the live 3-D image 20
focal distance 28
Focal length 50, 58
focus distance 50, 57
focused manually 18
frame number 65
frames per second 82
Gain 50
gray scale 20, 24
green bar 59, 65, 70, 84
Group 22, 51, 55, 65, 70, 73, 80, 83
Groups menu 86
Grow or Shrink 31
Grow or Shrink button 64
Height 25, 55
highlight non-consecutive images 70
highlight several consecutive images 70
Hologram 19, 25, 47, 60, 66, 84
holographic image color 42, 48, 80
Identify cells 44
Identify Cells 10
Identify Cells tab 29, 61
image color 55
image coloring 26
image dynamics 42, 48, 55, 80
image frame list 24
Image Frame list 32, 65, 70
image number 59, 70, 84
Information buttons 45
Join Nearby Markers 30, 64
Laser Pattern 19, 47

Light Effect 25, 42, 55, 80
Light Effect 20
Light Effect 47
Live Capture 44
Live Capture tab 18, 46
live holographic image display 46
Load button 32, 64
Main tabs 10
Main Viewing window 44
Manual 30, 63
manual changes 31
Manual Changes tab 64
Manual PART ONE 12
Manual PART TWO 17
manual software focusing 50
Measure button 34, 54
Medium ref index 51, 58
method 62
Method list 62
Minimum error 30, 63
Minimum Object Size 30, 64
Modify Location 74
Modify Location button 38
More 50
More list 58
move the cell image 24, 27
move the image 41, 80, 81
move the live image 20
Nbr 25, 41, 55, 80
New 70, 83
new color 48, 56, 81
new group 22
New Presets 32, 64
new project 22
number of cells 59, 65, 70, 84
Only checked 60
Only Checked 66, 84
Only Checked Frames 66
Otsu 30, 63
Otsu in blocks 30, 63
Perspective 80
Phase 19, 25, 47
Phase Contrast 60, 66, 84
Play 83
Presmoothing 30, 64
preview 43
Project 22, 55, 65, 70, 73, 79, 83
Project 51
Projects menu 86
Raw Image 33, 62

R-button 20, 26, 48, 81
R-button 55
Recalculate a holographic image 27
recalculate software focus 27
Recalibrate button 28, 58
redo 31
refractive indexes 58
Rel im center X 50
Rel im center X 58
Rel im center Y 50, 58
Remove 40, 69
Remove All command 36
Remove data from plot 36
Remove Highlighted command 36
Rename 70, 83
Rename button 32, 64
Revert 67
Rotate 19, 25, 47, 55
Save 76
save a colorset to an image 56
save a new colorset 49, 81
save a new colorset 56
Save Report 69
Save report button 36
scratch 89
Select 74
Select Cells 74
Select Mode 38
Set location 39, 74
Shiny surface 25, 42, 80
Shiny Surface 20, 47, 55
Show Cell Markers 33, 62
Show Color 25
Show Color Bar 20, 41, 47, 55, 79
Show Edge Cells Outline 33, 62
Show Histogram 62
Show Image Info 25, 41, 55, 79
Show Outline 33, 62
Show Ruler 20, 25, 41, 47, 55, 79
Show Threshold 33
Show Threshold 62
side windows 45
Side windows 11
Snapshot button 19, 24, 54
software focus 18
Software Focus 27, 56
software focus distance 27
software focusing, automatic 56
software focusing, manual 57
Source Frames 69

Source frames list 78
Source Frames list 73
Source Frames window 35, 37
Start the instrument 17
Startup 17
Stored Settings 32, 64
take number 59, 66
The Image Frame list 58
threshold 61
threshold settings, automatic 29
threshold settings, methods 29
threshold, adjust the 30
thumb nails 82
time lapse 22
timelapse 37
Timelapse 22, 52
Timeline 76
Timeline 38
Track 75
Tracked cells 73
Tracked cells list 78
tracks 38
Troubleshooting 89
Type 25, 41, 55, 80
Uncheck 66, 70, 84
Uncheck 59
Uncut 19, 47
undo 31
Undo for all Frames 39
Undo For All Frames 75
Undo for this Frame 39
Undo For This Frame 75
Unset 74
Unset button 39
Use buttons 41
well name 59, 66
Width 25, 55
View 86
View Images 10, 44
View Images tab 34, 53
Viewer Options 19, 33, 41, 46, 53, 61, 79
volume 68
X All 78, 83
X Highlighted 78, 83
X-axis 36
zoom 41
zoom the cell image 24, 27
zoom the image 80
zoom the live image 20